bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1 **Mucosal immunization with DTaP confers protection against *Bordetella pertussis* infection**
2 **and cough in Sprague-Dawley rats**
3
4 Jesse M. Hall[1,2], Graham J. Bitzer[1,2], Megan A. DeJong[1,2], Jason Kang[1,2], Ting Y. Wong[1,2], M.
5 Allison Wolf[1,2], Justin R Bevere[1,2] Mariette Barbier[1,2], & F. Heath Damron[1,2]
6
7 [1] Department of Microbiology, Immunology and Cell Biology, School of Medicine, West
8 Virginia University, Morgantown, West Virginia, USA
9 [2] Vaccine Development Center, West Virginia University Health Sciences Center, Morgantown,
10 West Virginia, USA
11
12 Running title: Mucosal immunization against *Bordetella pertussis* in rats
13
14 #Address correspondence to F. Heath Damron, fdamron@hsc.wvu.edu
15
16
17
18
19
20
21
22
23

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

24 **ABSTRACT**

25 Pertussis is a respiratory disease caused by the Gram-negative pathogen, *Bordetella pertussis* (*Bp*).
26 The transition from a whole cell pertussis vaccine (wP; DTP) to an acellular pertussis vaccine (aP;
27 DTaP; Tdap) correlates with an increase in pertussis cases, despite widespread vaccine
28 implementation and coverage, and it is now appreciated that the protection provided by aP rapidly
29 wanes. To recapitulate the localized immunity observed from natural infection, mucosal
30 vaccination with aP was explored using the coughing rat model of pertussis. Immunity induced by
31 both oral gavage (OG) and intranasal (IN) vaccination of aP in *Bp* challenged rats over a nine-day
32 infection was compared to intramuscular (IM)-wP and IM-aP immunized rats that were used as
33 positive controls as IM immunization is the current route for wP and aP vaccination. Our data
34 demonstrate that both IN and OG immunization of aP resulted in production of anti-*Bp* IgG
35 antibody titers similar to IM-wP and IM-aP vaccinated controls post-challenge. IN-aP also induced
36 anti-*Bp* IgA antibodies in the nasal cavity. Immunization with IM-wP, IM-aP, IN-aP, and OG-aP
37 immunization protected against *Bp* induced cough, while OG-aP immunization did not protect
38 against respiratory distress. Mucosal immunization (IN-aP and OG-aP) also protected against acute
39 inflammation and decreased bacterial burden in the lung compared to mock vaccinated challenge
40 (MVC) rats. The data presented in this study suggests that mucosal vaccination with aP can induce
41 a mucosal immune response and provide protection against *Bp* challenge.

42

43

44

45

46

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

47 **INTRODUCTION**

48 Infection of the respiratory mucosa by the Gram-negative bacterium *Bordetella pertussis* (*Bp*)
49 causes the disease known as pertussis (whooping cough) (1). Clinical manifestations of pertussis
50 are characterized by paroxysmal cough, hypertension, leukocytosis and in severe cases death,
51 particularly in infants who have yet to receive their first vaccine dose (2–4). Before pertussis
52 vaccines were introduced in the United States, pertussis led to approximately 200,000 deaths
53 annually (5). Largely, this disease has been under control by the use of diphtheria tetanus whole-
54 cell pertussis (DTP; wP) and acellular pertussis (DTaP; Tdap; aP) vaccines. DTP was first
55 introduced in the 1940s/1950s, and was largely effective in decreasing pertussis incidence (6). Due
56 to the robust immune response and reactogenicity concerns, developed countries converted to the
57 use of DTaP in the 1990s (2).
58
59 Since the introduction of the aP vaccine, pertussis cases have been increasing, despite high vaccine
60 coverage. It has been hypothesized that the increase in pertussis cases is attributed to: waning
61 immunity from DTaP and Tdap vaccination, vaccine driven evolution of *Bp* strains, increased
62 surveillance of pertussis, increased asymptomatic transmission, and improved PCR based
63 molecular identification of cases (7–13). Infant baboons vaccinated with DTaP (1 human dose at
64 2, 4, and 6 months of age) were still colonized after experimental *Bp* challenge in addition to
65 subsequent transmission to naïve baboons (14). In the same study, Warfel *et al* (2014) showed that
66 convalescent baboons that cleared a prior *Bp* infection, were not colonized following re-challenge
67 of *Bp* one month later (14). In humans, studies suggest that convalescent immunity can confer
68 protection for approximately 4-20 years (15), while DTaP immunity falls short lasting on average
69 4-12 years (15). Overall, these data demonstrated that immunity induced through natural infection

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

70 can generate longer lasting protection that also elicits pathogen clearance. As *Bp* is a respiratory
71 pathogen, it can be hypothesized that generating an immune response at the respiratory mucosa is
72 necessary for protection against pertussis.
73
74 Infection with *Bp* localizes to respiratory epithelium primes the immune response against
75 subsequent *Bp* infection by recruitment of antibody producing cells and tissue resident memory T
76 cells (Trm) (16). Bacteria invading mucosal surfaces can induce inflammation resulting in
77 subsequent production of IgA antibodies (17). Patients previously infected with *Bp* have developed
78 IgA antibody titers in their nasal secretions (18). In addition, anti-*Bp* IgA antibodies from patients
79 who have convalesced from *Bp* infection inhibit bacterial attachment *in vitro* and increase *Bp*
80 uptake and killing by human polymorphonuclear leukocytes (19, 20). Convalescent humans also
81 generate *Bp* specific IgG antibody titers that have shown to correlate with protection (21, 22). *Bp*
82 infected mice generate *Bp* specific CD4+ T cells in the lung that secrete IFN-γ and IL-17 (23, 24).
83 In mice, *Bp* infection induced Trms in the lung and were associated with pathogen clearance (16).
84 Nonhuman primates previously infected with *Bp* generate both Th1 and Th17 memory cells that
85 are still detected two years post infection (25). To induce a similar protective immune response
86 that is observed during natural infection, mucosal vaccination has recently been investigated (26–
87 28)
88
89 Mucosal immunization has been scarcely used as a vaccination strategy to generate a protective
90 immune response against pertussis in clinical and pre-clinical models. Oral vaccination with wP
91 induced the production of *Bp* specific antibody titers in both the saliva and serum of newborns
92 (29). In a subsequent study in 1985, oral immunization of 10^{12} CFUs of killed *Bp* led to the

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

93 induction of antibody titers in the saliva and serum of newborns (30). The frequency of pertussis
94 was lower in orally vaccinated newborns during the first year of life compared to newborns who
95 were unvaccinated although, this difference disappeared by the end of the year (30). In mice, oral
96 administration of attenuated bacterial vectors *Salmonella typhimurium* and *Escherichia coli*
97 expressing *Bp* antigen, filamentous hemagglutinin (FHA), results in the production of anti-FHA
98 IgA antibody titers in the lung (31). Intranasal immunization of a live attenuated vaccine strain of
99 *Bp,* BPZE1 was protective both in preclinical and clinical studies (32–35). Previously, our lab has
100 shown that IN immunization of DTaP can induce a protective immune response in mice (27, 28).
101 Boehm *et al* (2019) illustrated that IN vaccination of DTaP with and without the addition of the
102 adjuvant curdlan induces both anti-*Bp* and anti-pertussis toxin (PT) IgG antibody titers, as well as
103 *Bp* specific IgA in the lung (27). A subsequent study performed by Wolf *et al* (2021) suggested
104 that IN-aP vaccination is protective through the induction of humoral responses 6 months after
105 booster vaccination and challenge (28). Mounting evidence supports that mucosal immunization
106 can be protective against *Bp* colonization, but murine studies lack the ability to evaluate one the
107 of hallmark symptoms of pertussis, *Bp*-induced coughing.
108
109 The rat model of pertussis has been utilized to characterize *Bp* pathogenesis and evaluate coughing
110 manifestation from *Bp* infection (36–42). Only a few studies have been performed investigating
111 vaccine efficacy in the coughing rat model of pertussis. Hall *et al* (1998) demonstrated that the
112 SmithKline Beecham 3-component aP vaccine (detoxified PT (PTd), FHA, and a 69kDa antigen,
113 presumably pertactin (PRN)), the Connaught 5-component vaccine (PTd, FHA, agglutinins 2+3
114 (fimbriae), and PRN), and Evans whole-cell pertussis vaccine protected against cough upon
115 intrabronchial *Bp* challenge (41). Rats administered one single human dose of DTP had a lower

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

116 incidence of coughing following *Bp* challenge (39). We hypothesized that both oral and IN
117 vaccination would protect against bacterial burden upon challenge as well as protect against *Bp*
118 induced coughing in the rat model of pertussis by generating a protective immune response at the
119 site of infection. To test this hypothesis, we IN and OG vaccinated and challenged Sprague-
120 Dawley rats with 1/5th human dose of DTaP. IM-wP and IM-aP vaccinated and *Bp* challenged rats
121 were used as positive controls to compare vaccine-mediated immunity as IM administration of
122 DTaP is the current route of vaccine administration.

123

124 In our study, we aimed to use the rat model of pertussis to measure protection induced from
125 mucosal vaccination of DTaP. By utilizing the coughing rat model of pertussis, protection against
126 bacterial burden in the respiratory tract and prevention of *Bp* induced cough after immunization
127 was critically analyzed. We also focused on evaluating the serological responses regarding
128 vaccination followed by *Bp* challenge. Our data supports that protection can be afforded by
129 mucosal immunization with DTaP. IN and OG immunization with DTaP not only induced
130 systemic anti-*Bp* IgG antibodies but also induced mucosal anti-*Bp* IgA antibodies. These data
131 suggest that oral vaccination of DTaP can generate a humoral immune response at the respiratory
132 mucosa in rats. IN-aP and OG-aP was also capable of protecting against *Bp*-induced cough.
133 Furthermore, mucosal vaccination protected against bacterial burden in the respiratory tract. In
134 conclusion, this study highlights the benefits of using the coughing rat model of pertussis to study
135 mucosal vaccination with *Bp* vaccines.

136

137 **RESULTS**

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

138 **Intranasal immunization induces systemic anti-*Bp* IgG and anti-PT IgM and IgG antibody**
139 **titers following booster immunization.**
140 We hypothesized that IN-aP and OG-aP immunization would induce systemic IgM and IgG
141 antibodies, as mucosal immunization stimulates the induction of both systemic and mucosal
142 antibodies (27, 28, 30). To test this hypothesis, 3-week-old Sprague Dawley rats were IM-wP, IM-
143 aP, IN-aP, and OG-aP immunized followed by a booster vaccine at 6 weeks of age with the same
144 corresponding vaccine (**Fig. S1**). Mucosal and systemic antibodies were measured over the course
145 of vaccination (**Fig. S1**). Minimal differences in antibody titers prior to booster vaccination was
146 observed for all immunized groups (**Fig. 1**). Compared to all other groups, IM-wP vaccination
147 induced a significant increase of anti-*Bp* IgM in the serum 1-week post-booster vaccination (**Fig.**
148 **1A**). IM-wP, IM-aP, and IN-aP vaccinated rats had a significant increase in anti-*Bp* IgG antibody
149 titers following booster immunization compared to mock vaccinated challenged (MVC) rats (**Fig.**
150 **1B**). Rats vaccinated via IM-aP had a 100-fold significant increase in anti-PT IgM antibody titers
151 one week post booster vaccine, compared to the MVC control (**Fig. 1C**). IM-aP and IN-aP
152 vaccinated rats had a significant increase in anti-PT IgM antibodies compared to IM-wP, and OG-
153 aP vaccinated rats (**Fig. 1C**). This same trend was also observed in measuring anti-PT IgG
154 antibodies (**Fig. 1D**). Here our data show that following booster immunization, IN-aP vaccinated
155 rats developed systemic anti-*Bp* IgG and anti-PT IgG and IgM antibody titers.
156

157 **Mucosal vaccination protects against cough from *Bp* infected rats.**
158 In 2014, Warfel *et al* showed that aP vaccination was protective against pertussis disease, but failed
159 to protect against colonization and transmission of *Bp* in the nonhuman primate model (14). We
160 hypothesized that mucosal vaccination with DTaP would protect against *Bp* induced cough by

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

161 eliciting a protective immune response at the respiratory mucosa. To test this hypothesis,
162 vaccinated rats were subsequently intranasally challenged with *Bp* 2-weeks post-booster vaccine
163 administration (**Fig. S1**). Every evening post-challenge, coughs were counted using whole-body
164 plethysmography (WBP). MVC rats averaged a total of five coughs or less during monitoring for
165 the first 5 days of infection (**Fig. 2A**). At days 6 and 7 post-challenge, average coughs per fifteen
166 minutes increased to more than thirty coughs (**Fig. 2A**). There was a significant decrease in coughs
167 for rats vaccinated with IM-wP, IM-aP, IN-aP, and OG-aP at days 6 and 7 post-challenge compared
168 to MVC rats (**Fig. 2B-E**). On average, rats vaccinated with IM-wP coughed approximately 6
169 coughs per fifteen minutes each day (**Fig. 2B**). Rats vaccinated with IM-aP on average coughed 2
170 times per fifteen minutes each day (**Fig. 2C**). IN-aP immunized rats on average coughed 3 times
171 per fifteen minutes each day, while OG-aP vaccinated rats coughed on average 4 times per fifteen
172 minutes each day (**Fig. 2D&E**). To compare the average total number of coughs each day post-
173 challenge, we calculated the total number of coughs for each group per animal. We observed a
174 significant decrease in total number of coughs in rats vaccinated with IM-aP (14 coughs) and IN-
175 aP (27 coughs) compared to MVC rats (102 coughs) over the nine-day infection (**Fig. 2F**). OG-aP
176 immunized rats coughed on average 35 times (**Fig. 2F**). Our data demonstrates that mucosal
177 vaccination in rats protects against *Bp* induced cough.
178

179 **Intranasal vaccination protects against pulmonary distress.**

180 Our previous work has shown that *Bp* infected rats had a significant increase in pulmonary distress
181 following challenge (43). Pulmonary distress can be evaluated by calculating enhanced pause
182 (PenH). PenH functions as a representation of bronchoconstriction taking into consideration the
183 timing between early and late expiration and the estimated maximum inspiratory and expiratory

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

184 flow per breath. We hypothesized that mucosal vaccination would protect against *Bp* induced
185 pulmonary distress, as bacterial clearance would decrease inflammation. Here, rats vaccinated with
186 IM-wP, IM-aP, and IN-aP had a significant decrease in PenH compared to the MVC control group
187 at days 5 and 7 post-challenge (**Fig. 3A-D**). Rats vaccinated IM-aP and IN-aP also had a significant
188 decrease in PenH at day 6 post-challenge compared to MVC rats (**Fig. 3A&C-D**). However, there
189 was no significant decrease in PenH in OG-aP vaccinated rats compared to MVC suggesting that
190 the induced immune response is not sufficient enough to protect rats from *Bp* induced respiratory
191 distress (**Fig. 3E**). Other respiratory parameters were also measured using WBP (**Fig. S2**). In brief,
192 we observed that rats vaccinated IM-wP, IM-aP, and IN-aP had a significant decrease in pause
193 (PAU) compared to MVC control group, which is another indicator of bronchiole restriction (**Fig.**
194 **S2B**). Rats vaccinated with the aP regardless of route also had a significant decrease in Tidal
195 volume (TVb) compared to MVC rats, which could be crudely associated with inflammation (**Fig.**
196 **S2D**). Overall, our data demonstrated that IN-aP vaccination decreases pulmonary distress of *Bp*
197 infected rats.
198
199 **Mucosal immunization induces production of *Bp* specific antibodies in the serum, while**
200 **intranasal immunization also induces PT specific antibodies in the serum.**
201 Next, we wanted to measure systemic antibody responses to *Bp* and PT following challenge. IM-
202 wP vaccinated rats had a slight increase in anti-*Bp* IgM antibodies compared to all other vaccinated
203 groups, albeit not significant (**Fig. 4A**). We observed a significant increase in anti-*Bp* IgG antibody
204 titers in IM-wP, IM-aP, and IN-aP vaccinated rats compared to the MVC at day 1 post challenge
205 (**Fig. 4B**). At day 9 post-challenge, all vaccinated rats had a significant increase of anti-*Bp* IgG
206 antibody titers compared to the MVC control (**Fig. 4B**). Following *Bp* challenge, IM-aP and IN-

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

207 aP immunized rats had a significant increase in anti-PT IgM antibodies compared to IM-wP
208 immunized rats and MVC control. (**Fig. 4C**). Similar results were observed in measuring anti-PT
209 IgG titers (**Fig. 4D**). IM-aP and IN-aP vaccination induced a significant increase in anti-PT IgG
210 antibody titers compared to MVC, IM-wP, and OG-aP immunized rats after booster vaccination
211 and at days 1 and 9 post-challenge (**Fig. 4D**). Although not significant, two of the OG-aP
212 immunized rats had detectable anti-PT IgG antibody titers in the serum at day 9 post-challenge
213 (**Fig. 4D**). Enzyme-linked immune absorbent spot (ELISpot) assay was used to determine the
214 number of *Bp* specific IgG cells in the bone marrow at day 9 post-challenge. There was an increase
215 in the number of *Bp* specific IgG cells in the bone marrow in all vaccination groups compared to
216 the MVC control; however, the only significant increase in *Bp* specific IgG producing cells in the
217 bone marrow was detected in IM-wP vaccinated rats (**Fig. S3**). Our data indicate that mucosal
218 vaccination via IN and OG immunization induced *Bp* specific IgG antibody responses.
219
220 **Intranasal immunization induces production of *Bp* specific IgA antibodies in the nasal cavity**
221 In humans, previous *Bp* infection leads to anti-*Bp* IgA antibodies in nasal secretions (18). IgA
222 antibodies to *Bp* play a role in the inhibition of *Bp* attachment *in vitro* to epithelial cells (19). Here,
223 we investigated if IN and OG immunization of DTaP would induce mucosal IgA antibodies in the
224 lung and/or the nasal cavity. In the lung, three of the four IN-aP vaccinated rats had detectable
225 anti-*Bp* IgA antibodies at day 1 post-challenge, although not significant (**Fig. 5A**). We did not
226 detect anti-*Bp* IgA antibody titers at day 1 post-challenge in the lung of IM-wP, IM-aP, or OG-aP
227 vaccinated rats (**Fig. 5A**). Low levels of anti-*Bp* IgA titers were measured in all vaccinated groups
228 at day 9 post-challenge albeit not significant compared to our MVC control (**Fig. 5A**). The same
229 trend was observed in the lung measuring anti-PT IgA titers at day 1 post-challenge (**Fig. 5B**). At

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

230 day 9 post-challenge 50% of IN-aP rats and 25% of rats OG-aP vaccinated had detectable anti-PT
231 IgA (**Fig. 5B**). In the nasal cavity, only one IN-aP vaccinated rat had detectable anti-*Bp* IgA
232 antibody titers; however, we did measure a significant increase in anti-*Bp* IgA antibodies in IN-aP
233 immunized rats at day 9 post-challenge compared to the MVC, IM-wP, IM-aP, and OG-aP
234 immunized rats (**Fig. 5C**). Only one IN-aP and one OG-aP vaccinated rat had detectable amounts
235 of anti-PT IgA in the nasal cavity at day 9 post challenge (**Fig. 5D**). Overall, our data reveal that
236 IN-aP immunization is capable of inducing IgA antibodies in the nasal cavity of rats.
237
238 **Mucosal immunization protects against acute inflammation in the lung.**
239 Our previous rat challenge study illustrated that intranasal *Bp* challenge gave rise to both acute and
240 chronic inflammation in the rat lung (43). Here, we used histology to assess if mucosal
241 immunization would protect against *Bp* induced inflammation in the lung. At day 1 post-challenge,
242 no differences in acute inflammation were observed; however, at day 9 post-challenge, rats
243 vaccinated with IM-aP, IN-aP, and OG-aP had a significant lower acute inflammation scoring
244 compared to the MVC rats. (**Fig. 6A&C**). IN-aP immunized rats had a higher chronic
245 inflammatory score at day 1 post-challenge (**Fig. 6B&D**). There were no observed differences in
246 chronic inflammation in vaccinated rats compared to MVC rats at day 9 post-challenge (**Fig. 6D**).
247 Total inflammation was calculated by combining both acute and chronic inflammatory scores, as
248 rat lungs exhibited both types of inflammation. No differences in total inflammation score were
249 observed at day 1 post-challenge; however, all vaccinated rats had a significant lower total
250 inflammation score compared to MVC rats (**Fig. 6E**). There were no differences in lung weight,
251 which can be used as a crude measurement for lung inflammation, following *Bp* challenge. (**Fig.
252 S4A**). We did observe a significant increase in percent body weight change in IM-wP vaccinated

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

253 rats compared to MVC control rats suggesting that wP protects against weight loss observed in
254 non-vaccinated challenged rats (**Fig. S4B**). Our observations suggest that that mucosal vaccination
255 protects against *Bp* induced inflammation in the lung (**Fig. 6C&E**).
256
257 **Mucosal vaccination protects against *Bp* challenge.**
258 Next, we wanted to assess if mucosal immunization could protect against *Bp* burden in the
259 respiratory tract. Bacterial burden in the respiratory tract was determined 1hr, 1-, and 9- days post
260 *Bp* challenge. Bacterial burden was measured at 1hr post-challenge (n=2) to assess potential
261 bacterial loss for our original challenge dose. In the lung, trachea, and nasal lavage fluid, we
262 measured approximately 10^6 CFUs 1hr post challenge (**Fig. 7A-C**). At day 1 post-challenge there
263 was a significant 98.5% reduction in bacterial burden in the lung of IM-aP immunized rats
264 compared to MVC (**Fig. 7A**). IM-wP. IM-aP, IN-aP, and OG-aP vaccinated rats all had a
265 significant decrease in bacterial burden in the lung at day 9 post-challenge compared to MVC rats
266 (**Fig. 7A**). There was also a significant decrease in bacterial burden in the trachea at both days 1
267 and 9 post-challenge in all vaccinated rats compared to MVC (**Fig. 7B**). At day 1 post-challenge,
268 there was a significant 86-97% reduction in bacterial burden in all vaccinated rats compared to
269 MVC rats in the nasal cavity (**Fig. 7C**). At day 9 post-challenge we did not measure any significant
270 differences between groups as most of the bacteria were cleared from the nasal cavity (**Fig. 7C**).
271 Overall, we observed that IN-aP and OG-aP vaccinated rats have a significant reduction in
272 bacterial burden in the respiratory tract compared to the MVC control group at days 1 and 9 post-
273 challenge (**Fig. 7A-C**).
274

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

275 **wP immunization induces a proinflammatory cytokine response compared to mucosal**
276 **vaccinated Sprague-Dawley rats.**

277 Previous studies have shown that both wP immunization and *Bp* infection induces a pro-
278 inflammatory Th1/Th17 immune response, while aP immunization promotes a more Th2 skewed
279 response (44–50). In our current study, we measured cytokines in the lung and serum induced from
280 vaccination and challenge. In the lung at day 1 post-challenge, we measured a significant 4-fold
281 increase in IL-17 in MVC rats compared to IM-aP and IN-aP vaccinated rats (**Fig. 8A**). At day 9
282 post-challenge, IM-wP immunized rats had a significant increase in IL-17 compared to IM-aP, IN-
283 aP, and MVC rats (**Fig. 8A**). IM-wP vaccinated rats also had a significant increase in Th1 cytokine
284 IL-12p70 compared to MVC rats in the lung and serum at day 9 post-challenge (**Fig. 8A&B**). IM-
285 wP vaccinated rats had a significant increase in Th2 cytokines IL-4 and IL-13 in the serum
286 compared to IM-aP and IN-aP vaccinated rats and a significant increase in IL-4 to MVC control
287 at day 9 post-challenge (**Fig. 8B**). IM-wP immunized rats also had a significant increase in G-CSF
288 in the serum day 9 post-challenge compared to IM-aP, IN-aP, OG-aP, and MVC rats. Overall, we
289 did not observe marked changes in cytokine responses between DTaP vaccinated rats compared to
290 the MVC control group; however, rats OG-aP immunized did have a slight increase in IL-17,
291 though not significant. The observed difference in cytokines levels could be from *Bp* challenge
292 rather than vaccination. The increase in acute inflammatory score in the lung of IM-wP immunized
293 rats at day 9 post-challenge could be associated to the increase in proinflammatory cytokines.
294 Graphs showing the statistical significance between cytokines in the serum and lung are in the
295 supplementary data (**Fig. S5&6**). Our data support that response to *B. pertussis* in IM-wP
296 immunized animals is associated robust cytokine response compared to both naïve and aP

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

297 vaccinated rats, which is to be expected based on the work that has examined the Th17 response
298 induced by whole cell pertussis vaccines.
299
300 *Bp* infection induces an increase in circulating neutrophils in the blood, as well as white blood
301 cells and lymphocytes (51–56). In our current study, we utilized hematology and flow cytometry
302 to evaluate these populations. Hematology analysis revealed a significant increase in blood
303 lymphocytes in the IN aP vaccinated rats compared to MVC post-challenge; however, no other
304 differences in white blood cell counts in the blood were observed in the other vaccinated groups.
305 (**Fig. S7A&B**). At day 1 post-challenge, there was a significant decrease in circulating neutrophils
306 in the blood for IN-aP immunized rats compared to MVC rats (**Fig. S7C**). Flow cytometry analysis
307 observed minimal differences in the number of neutrophils and B cells at days 1 and 9 post-
308 challenge in all groups (**Fig. S7E&F**). Based upon these data, subtle differences in various
309 circulating cell populations were observed following IN-aP vaccination.
310
311 **Serological responses correlate with bacterial clearance in the respiratory tract.**
312 Currently no definitive correlates of protection (CoP) for vaccines to protect against *Bp* have been
313 established (22). It is appreciated that Th17 responses as well as Trms correlate with strong
314 protection in mice and baboons. Antibodies to PT/FHA/PRN do not always correlate with
315 protection in humans. In an effort to more precisely define correlates, using the rat model, we
316 aimed to utilize the coughing phenotype and bacterial burden to identify the nature of how each
317 vaccine protects (OG/IN/IM; acellular or wP). Previous work in our lab performed by Wolf *et al*
318 (2021) illustrated that serum anti-*Bp*, anti-FHA, and anti-PT IgG antibody titers in the serum
319 following IN vaccination in mice correlate with the decrease in bacterial burden in the lung
320 following *Bp* challenge (28). Previous studies have shown that serum anti-*Bp* IgG antibodies

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

321 induced from wP vaccination correlate with protection against bacterial burden in the lung of *Bp*
322 challenged mice (57). Here, we hypothesized that antigen specific serum IgG and mucosal IgA
323 antibodies correlate with decreased bacterial burden and cough, as IN-aP and OG-aP vaccination
324 induced systemic and mucosal antibody responses. To test this hypothesis, we generated
325 correlograms to evaluate both negative and positive correlations elicited by each vaccination route
326 (58). Correlograms are an analysis tool that can be used to determine if the relationship observed
327 between variables (i.e. bacterial burden and antibody titers) is random or not (59). If the
328 relationship between the two variables is random the R^2 correlation value is or near zero (59). The
329 relationship is considered correlative if the R^2 values approximately positive or negative one (59).
330 Significant positive nonzero correlation values demonstrate a positive correlation between
331 variables, while significant negative nonzero values represent a negative correlation (59). Negative
332 correlations are observed when two variables are inversely related to one another; that is, when
333 one variable increases, the other decreases. With these data, as bacterial burden would drop, then
334 the correlate would increase (negative correlation; inverse). A positive correlation would mean
335 that as bacterial burden increases so does the correlate that is being compared to.
336
337 IM-wP vaccinated rats had strong negative correlations (protective) between serum anti-*Bp* IgG
338 antibodies to both bacterial burden in the lung (R^2= -0.97) at day 1 post challenge and the nasal
339 cavity (R^2 = -0.84) at day 9 post-challenge (**Fig. 9A-B**). At day 1 post-challenge, we observed
340 negative correlations (protective) between systemic anti-*Bp* IgM and anti-PT IgG antibodies in
341 IM-aP vaccinated rats to bacterial burden in the nasal cavity (R^2 = -0.64, -0.64 respectively).
342 Additionally, negative correlations were observed between anti-*Bp* IgM antibodies and bacterial
343 burden in the trachea (R^2 = -0.72) at day 9 post-challenge (**Fig. 9C-D**). IM-aP vaccinated rats also

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

344 had negative correlation between total cough counts over the course of challenge with IgG and
345 IgM antibodies to *Bp* and PT (**Fig. 9D**). We expected that IN immunization would induce negative
346 correlations between both serum- and mucosal-specific antibodies compared to bacterial burden
347 in the lung, trachea, and nasal cavity at day 1 post-challenge (**Fig. 9E**). Lung anti-*Bp* IgA
348 antibodies also negatively correlated with total cough count (R^2 = -0.74) and bacterial burden (R^2
349 = -0.6) in the lung at day 9 post-challenge for IN-aP vaccinated rats (**Fig. 9F**). We observed strong
350 negative correlations between serum IgG and mucosal IgA antibody to bacterial burden in the lung
351 (R^2 = -0.93, -0.93 respectively) in OG-aP vaccinated rats at day 1 post-challenge despite the overall
352 lower serological responses (**Fig. 9G**). At day 9 post challenge, there was a negative correlation
353 between serum anti-*Bp* IgG antibodies to bacterial burden in the lung (R^2 = -0.81), trachea (R^2 = -
354 0.49), and nasal cavity (R^2 = -0.52) in OG-aP immunized rats (**Fig. 9H**). We also noticed that OG-
355 aP vaccinated rats had negative correlations between serum IgG and mucosal IgA antibodies in
356 the lung to total cough count (R^2 = -0.94, -0.84) at day 9 post-challenge (**Fig. 9H**). Positive
357 correlations (non-protective) were observed between bacterial burden and total inflammatory score
358 in IM-wP, IM-aP, and OG-aP immunized rats (**Fig. 9A, C, G**). Bacterial burden also positively
359 correlated with total cough counts at day 9 post challenge (**Fig. 9B, D, F, H**). By utilizing
360 correlograms, strong negative correlations between serum serological responses and bacterial
361 burden were observed in IM-wP and IM-aP immunized rats. Additionally, our data underscore
362 the idea that both systemic and mucosal antibodies correlate with the observed *Bp* clearance in the
363 respiratory tract and protection from *Bp* induced cough elicited from IN-aP and OG-aP vaccination
364 highlighting the observed differences between vaccination routes.
365

366 **Discussion**

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

367 The immunity induced by aP vaccines is relatively short lived; thus, DTaP/Tdap vaccinated
368 individuals are still capable of *Bp* transmission (60, 61). We have recently re-investigated the rat
369 model of pertussis to further understand *Bp* pathogenesis from current circulating *Bp* strains (such
370 as CDC isolate D420) (43). The coughing rat model of pertussis is a tool that can be used to
371 evaluate bacterial burden in the respiratory tract, and also evaluate vaccine-induced immunity
372 against cough and respiratory function (36–41, 62). In our current study, we evaluated mucosal
373 vaccination with DTaP in the coughing rat model of pertussis. To our knowledge, this study is
374 the first to evaluate both IN and OG administered DTaP in the coughing rat model of pertussis.
375 The data presented here suggest that, not only does mucosal immunization protect against bacterial
376 burden, but also against *Bp* induced cough by WBP (**Fig. 2&7**).
377
378 Vaccine mediated immunity has been studied in the coughing rat model of pertussis. Utilizing
379 audio tape recorders, Hall *et al* (1998) illustrated that 3 and 5 component aP vaccines administered
380 subcutaneously could protect against *Bp* induced cough (41). wP immunization administered
381 intraperitoneally also decreased the incidence of cough in rats (39). Neither study noted protection
382 against bacterial colonization. Here, in our study, WBP was used to investigate *Bp* induced cough
383 in IN-aP and OG-aP vaccinated rats, as well as measure bacterial burden in the respiratory tract.
384 Our data supports that mucosal administration of DTaP protects against bacterial burden in the
385 respiratory tract and reduced *Bp*-induced cough (**Fig. 2&7**). In addition, IN-aP vaccination reduced
386 bronchial constriction in the lung that is elicited by *Bp* infection (**Fig. 4**). Previous studies
387 evaluating wP and aP vaccines in the rat model of pertussis used one human dose per rat for
388 immunization prior to challenge (39, 41). In an effort to best model an appropriate human to rat
389 dose, we utilized a 1/5th human dose to prime and boost based on the relative sizes of rats compared

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

390 to mice. We have reported 1/40th human dose as protective in mice and rats are roughly 10x the
391 weight of mice (63). One caveat of this study is that we did not evaluate other human-to-rat
392 titrations of aP. Identification of a minimal protective rat dose would allow for the investigation
393 of vaccine efficacy of new potential antigens/adjuvants in this model (63).
394
395 Mucosal immunization has been of particular interest in the pertussis field. We and others have
396 recently evaluated intranasal immunization of DTaP in *Bp* challenged mice (27, 28, 64–66).
397 Intranasally DTaP vaccinated mice were protected against *Bp* challenge and also generated both
398 systemic and mucosal antibodies (27, 28). Live attenuated strain BPZE1 administered intranasally
399 was protective against *Bp* challenge in mice and baboons, and is currently in Phase 2 of clinical
400 studies (33, 34, 67, 68). BPZE1 immunization induces both anti-*Bp* IgG and IgA antibodies
401 systemically and has an increase in resident memory T cells in the lung (33, 69). Oral immunization
402 has also been investigated as a possible vaccination strategy against pertussis. Oral immunization
403 of heat-inactivated *Bp* protected newborns against *Bp* challenge, as well as generated serum and
404 saliva antibody titers (70). Recombinant technologies has led to the development of live attenuated
405 *Salmonella* strains presenting *Bp* antigens (71, 72). Oral immunization of *Salmonella typhimurium*
406 *aro* vaccine strain harboring the gene for PRN resulted in reduced bacterial colonization in the
407 lung post *Bp* challenge (71). *Salmonella dublin aroA* mutant expressing the gene for FHA was
408 also orally administered as a vaccine in mice (72). Vaccination with this strain induced IgG and
409 IgA antibody titers to FHA in the serum and gut (72). Our current study shows that mucosal
410 immunization not only induced systemic anti-*Bp* IgG but also anti-*Bp* IgA antibodies that likely
411 play role in clearance at the mucosa (**Fig. 4-5, 7**).
412

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

413 CoP is defined as the immune response that is statistically accountable for the observed protection
414 (73). While no CoP has yet to be fully agreed upon against pertussis in humans, anti-PT IgG levels
415 >5 IU/ml are associated with protection in humans (74). In mice, IN administration of DTaP
416 induced anti-*Bp*, anti-FHA, and anti-PT IgG antibodies while wP vaccination induced serum anti-
417 *Bp* IgG antibodies that correlated with protection against *Bp* (28, 57). Here in our study, we
418 generated correlograms between all vaccinated groups to identify correlations between variables
419 in the coughing rat model of pertussis, which has yet to be established (**Fig. 9**). Our results indicate
420 that bacterial clearance in the lung, trachea, and nasal cavity negatively correlate with systemic
421 anti-*Bp* and -PT IgM and anti-*Bp* IgG antibodies in IM-wP and IM-aP immunized rats, while
422 systemic and mucosal antibodies correlated with bacterial clearance in IN-aP and OG-aP
423 vaccinated rats (**Fig. 9**). Antibodies generated following immunization also negatively correlated
424 with a decrease in total cough counts in vaccinated rats (**Fig. 9**). These results suggest that the
425 increase in systemic and mucosal antibodies induced from IN and OG vaccination correlates with
426 protection against *Bp* burden in the respiratory tract and *Bp* induced cough. It is important to note,
427 that OG-aP immunized rats did not generate significant serum antibody titers to the whole
428 bacterium until day 9 post-challenge (**Fig. 4B**). Also, two OG-aP immunized rats had an increase
429 in anti-PT IgG antibody titers in the serum and anti-*Bp* IgA antibodies in the lung (**Fig. 4D, 5A**).
430 We did however detect antigen specific B cells in the bone marrow and 3 of the 4 rats had low
431 levels of IgA in the lung in OG-aP immunized rats (**Fig. S3**). One caveat that should be mentioned
432 is that we did not investigate the T cell responses (Th1/Th2/Th17/Trm/Tem) in rats but future
433 studies will incorporate this into the study design. We have proposed a summary for mechanism
434 for oral vaccination of aP (**Fig. S9**). We hypothesize that this could be because limited amount of
435 vaccine that successfully travels to the gut-associated lymphoid tissue (GALT) for the generation

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

436 of an immune response. Oral vaccines have to travel through increased pH in the stomach while
437 limited absorption and availability for antigen recognition also occur in the gastrointestinal tract
438 (75). Increase in dose, number of doses, or delivering vaccine in an encased vehicle are potential
439 methods to increase orally vaccinated immune responses. Targeting of vaccine to intestinal M cells
440 for antigen presentation has also been shown to increase oral vaccine efficacy (76). Though we
441 did not study new adjuvants here, we hypothesize adjuvants can aid in stimulating a protective
442 mucosal immune response. These approaches could all potentially increase the efficacy observed
443 through oral vaccination of aP. Furthermore, to deliver the vaccine to the gut, one could envision
444 novel deliver mechanisms such as gelatin coated chewables similar to gummy vitamins that are
445 now popular.
446
447 In summary, mucosal vaccination not only protected against bacterial burden in the respiratory
448 tract of challenged rats, but also protected against *Bp* induced cough and respiratory distress
449 measured by WBP (**Fig. 2-3, 7**). It is critical that "next generation pertussis" vaccines protect
450 against bacterial colonization in the lung, nasal cavity, and trachea, as disease manifestations are
451 dependent on bacterial colonization of the lung and trachea, mediated by FHA and fimbriae (77,
452 78). Both IN and OG immunized rats generated anti-*Bp* specific IgG antibodies in the serum, while
453 IN vaccinated also generated significant anti-*Bp* IgA antibody titers in the nasal cavity following
454 challenge (**Fig. 4-5**). IN-aP and OG-aP immunized rats were protected against acute and total
455 inflammation in the lung (**Fig. 6**). Our data support the potential of a mucosal vaccination against
456 *Bp.*
457

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

458 Further work is needed to fully characterize the immune response generated following IN-aP and
459 OG-aP vaccination in rats, as well as vaccine mediated immunity from vaccination in the coughing
460 rat model of pertussis. T cell immune responses that have been shown to play a role in natural and
461 vaccine mediated immunity against pertussis have yet to be evaluated in the coughing rat model
462 of pertussis due to limited availability of resources to adequately measure T cell responses.
463 Vaccine mediated memory has yet to be evaluated in the coughing rat model of pertussis.
464 Additional research is needed to critically assess vaccine mediated memory, as it is essential that
465 next generation of pertussis vaccines induce longer lasting memory then current vaccines. Future
466 work is also needed to evaluate mucosal immunization against current circulating strains of *Bp* as
467 current strains are genetically divergent from strains of the past, with the goal of making the most
468 efficacious vaccine against *Bp*.
469

470 **MATERIALS AND METHODS**

471 **Vaccine composition and administration.** INFANRIX (GSK Cat. 58160-810-11) acellular
472 pertussis human vaccine (DTaP) and the National Institute for Biological Standards and Control
473 WHO whole cell pertussis vaccine (NIBSC code 94/532) was used for this study. Vaccines were
474 diluted with endotoxin-free Dulbecco's PBS (Thermo Fisher Scientific Cat. TMS012A) to a
475 concentration of 1/5th human dose. Vaccines were diluted and administered no more than 1 hr.
476 from composition. The first dose of vaccine was administered to three-week-old (50g) female
477 Sprague-Dawley rats (Charles River Cat. 001CD). At six weeks of age, a booster vaccine of the
478 same dose was administered, followed by *Bp* challenge at eight weeks of age. Intramuscular (IM)
479 vaccinated rats received 100µl in the right thigh muscle of the hind limb. Intranasal (IN)
480 immunized rats were first anesthetized with isoflurane until breathing was minimal. Rats then

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

481 received 50µl of vaccine in each nostril for a 100µl dose. Oral gavage (OG) vaccinated rats
482 received 100µl dose delivered curved 18 gauge feeding needle (Fisher Scientific Cat.
483 NC9349775). MVC control group received 100µl of the same endotoxin free PBS used to dilute
484 the vaccines in the right thigh muscles of the hind limb. One-week post-prime, two-week post-
485 prime, and one-week post-boost blood was collected via saphenous blood draws for serological
486 analysis. 5mm animal lancets (Fisher Scientific Cat. NC9891620) was used for blood draw. Blood
487 was collected in capillary tubes (Fisher Scientific Cat. NC9059691) for centrifugation. Blood was
488 spun at 15,000x *g* for 3 min., serum collected and stored at -80˚C until analysis.
489

490 ***Bordetella pertussis*** **strains and growth conditions.** *Bp* strain D420 was cultured on Bordet
491 Gengou (BG) agar (Remel™ Cat. R45232) supplemented with 15% defibrinated sheep blood
492 (Hemostat Laboratories Cat. DSB500) (1). Bacteria cultured BG plates incubated for 48 hrs at
493 36˚C. Using polyester swabs (Puritan Cat. 22-029-574), *Bp* was transferred into 20 ml Stainer-
494 Scholte liquid media (SSM) in new 125 ml flasks (Thermo Fisher Scientific Cat. FB500125) (79).
495 Bacterial cultures were allowed to grow at 36˚C for 24 hrs inside a shaking incubator at 180 rpm.
496

497 **Intranasal challenge.** Vaccinated eight-week-old ~200g female Sprague-Dawley rats were then
498 challenged. *Bp* was grown as illustrated above. Rats were anesthetized with ketamine and xylazine
499 50-100/5-10 mg/kg and challenged with 10^8 CFUs in 100µl intranasally, 50µl in each nostril. Body
500 weight of each rat was recorded before bacterial challenge, and body weights were taken post-
501 euthanasia to calculate percent weight change. At days 1 and 9 post challenge, rats were then
502 euthanized. Upon euthanasia blood was collected via cardiac puncture and transferred into
503 ethylenediaminetetraacetic acid (EDTA) (BD Cat. 365974) and serum separation (BD Cat.

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

504 026897) tubes. Following cardiac puncture, 250µl of blood was collected into EDTA tubes for
505 flow cytometry and ProCyte (IDEXX) analysis, while remaining blood was collected in serum
506 separation tubes to isolate the serum via centrifugation (15,000x *g* for 3 min) and used for
507 serological and cytokine analysis. To determine bacterial burden in the respiratory tract, the lung
508 and trachea was excised separately and homogenize. Lung weights were recorded following
509 excision before homogenization. Lungs were then collected in gentleMACS C tubes (Miltenyi
510 Biotec Cat. 130-096-334) in 2ml of PBS and homogenized using Miltenyi Biotec tissue dissociator
511 (Cat. 130-095-927). Polytron homogenizer was used to homogenize the trachea in 1 ml PBS.
512 Bacterial burden in the nares was determined by flushing 2mls of sterile 1x PBS through the nares
513 and collected for serial dilution and plating. Serial dilutions of the homogenates and nasal
514 collection were plated on BG plates supplemented with ceftibuten (Sigma-Aldrich Cat. SML0037)
515 10 µg/ml.

516 **Serological analysis.** Enzyme-linked immunosorbent assays (ELISA) was used to measure
517 antibody titers of vaccinated and infected rats. *Bp* specific whole bacteria ELISA plates were
518 coated with 50 µl of 10^8 *Bp* grown as mentioned above for infection. Antigen specific antibody
519 titers to PT (List Biological Laboratories #180) were measured by coating ELISA plates with 50
520 µl of antigen per well. Antigen coated plates incubated over night at 4°C. After incubation, plates
521 were washed with 1x PBS-Tween 20 and blocked with 5% skimmed milk for 2 hrs at 37°C.
522 Following blocking, ELISA plates were washed and serum from the saphenous blood draws and
523 blood collected from cardiac puncture post-euthanasia were serially diluted down the ELISA plate
524 and incubated for 2 hrs at 37°C. To measure respiratory IgA antibody titers in the lung and nasal
525 lavages, lung homogenate supernatant and nasal lavage was added and incubated for 2 hrs at 37°C.
526 After incubation, ELISA plates were washed as described above and 100µl of secondary goat anti-

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

527 rat IgG (SouthernBiotech Cat. 3030-04), goat anti-rat IgM (SouhternBiotech Cat. 3020-04), or
528 goat anti-rat IgA (MyBioSource Cat. MBS539212) was added to the plates at a dilution of 1:2,000
529 in PBS + 5% milk and incubated for 1 hr at 37°C. Plates were then washed again and 100 µl *p*-
530 nitrophenyl phosphate substate (Thermo Scientific Cat. 37620) was added and the plate was
531 developed for 30 min at room temperature. After development, colorimetric signal of the ELISA
532 plate at A_{450} was measured by a Biotek Synergy H1 microplate reader. Antibody titers were
533 considered positive if values were higher than the baseline. Baseline value for each sample was
534 set as double the average value of the blank, in which no serum, lung supernatant, or nasal lavage
535 added to these well. Limit of detection was set at 50, and any samples with a titer value less than
536 that were set to 50.

537 **ELISpot assay.** ELISpot assay (ImmunoSpot Cat. mTgG-SCE-1M/2) was used to analyze antigen
538 specific B cells in the bone marrow. The right hind femur of the rat was removed and placed into
539 Dulbecco's modified Eagle's medium (DMEM) and frozen at -80°C until analysis. Bones were
540 then thawed in water bath at 37°C, and immediately transferred into spin tubes and spun at 1,000x
541 *g* for 3 min to collect the bone marrow. Bone marrow was passed through a 70µm filter to create
542 a single cell suspension. Cells were centrifuged at 350 x *g* for 5 min and the cell pellet was
543 resuspended in CTL test B Media (ImmunoSpot). D420 was cultured as described above and
544 coated the 96-well ELISpot plate as described by ELISA. The plate incubated overnight at 4°C.
545 Plate was then washed with 1x PBS before cells were added. Three serial dilutions of cells (1.25
546 x 10^6, 3.13 x 10^5, and 1.56 x 10^5) cells added per well and incubated at 37°C overnight. Rabbit
547 anti-Rat IgG antibody (Abcam Cat. ab6733) was used to replace the anti-murine IgG detection
548 antibody that was with the kit. The rest of the protocol was followed as per the manufacturer's

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

549 instructions. ELISpot plates imaged and analyzed using ImmunoSpot S6 Entry analyzer and CTL
550 software.
551 **Analysis of cough and bronchiole restriction using whole-body plethysmography.** Buxco®
552 FinePointe^{TM} Whole Body Plethysmography (WBP) (DSI) was used to quantify respiratory
553 function during infection. Every day following *Bp* challenge and one day before challenge
554 (5:00PM), rat respiratory profiles and coughs were measured. A 5 min acclimation period was
555 used before measuring cough and other respiratory parameters. After acclimation the respiratory
556 profile was recorded for 15 mins for each rat. Coughs were counted and represented over 15 mins.
557 Enhanced pause (PenH) was calculated which represents bronchiole restriction during breathing.
558 Coughs were counted based on box flow changes of the subject with classical cough-like
559 waveforms. Patented fuzzy logic criteria was used to detect and count coughs (80). Each cough in
560 a multi-cough event was counted individually. Frequency (F), Tidal Volume (TVb), Pause (PAU),
561 Minute Volume (MVb), Inspiratory time (Ti), and expiratory time (Te) were also collected and
562 analyzed during the course of infection.
563 **Histological assessment of the lung.** The left lobe of the lung was used for histological
564 assessment. Following excision of the left lobe, the sectioned portion was fixed in 10% formalin
565 48 hrs at 26 °C. Following fixation, samples were embedded in paraffin and stained with H&E by
566 the WVU Pathology Department. Stained samples were used to characterize and score
567 inflammation of the lung. All scorings were done by a board-certified pathologist (iHisto).
568 Individual scores were based on a standard qualitative scoring criterion: (0 – none, 1 – minimal
569 (rare), 2 – mild (slight), 3 – moderate, 4 – marked, 5 –severe). The presence of neutrophils in the
570 parenchyma, blood vessels, and airway was used to score acute inflammation, and chronic

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

571 inflammation was characterized by mononuclear infiltrates of the parenchyma, blood vessels, and
572 airway. All examination and scoring were done with no knowledge of the groups.

573 **ProCyte analysis of blood.** Blood from the EDTA tubes was used to analyze white blood cell,
574 neutrophil, and lymphocyte counts. 25-50µl of blood was drawn from the EDTA tubes and
575 analyzed by the Procyte. After ProCyte analysis the rest of the blood was used for flow cytometry
576 analysis.

577 **Flow cytometry analysis.** Blood samples were lysed with 1x Pharmylse buffer (BD Biosciences
578 Cat. 555899) for 20 min at room temperature. Blood samples were vortexed periodically during
579 the 20 min incubation. After lysis, cells were resuspended in RPMI + 10% FBS to neutralize the
580 lysis buffer and centrifuged at 1,000g x 5. Cells were then washed with the RPMI +10% FBS
581 again. Cells were then resuspended in 1%FBS+PBS+5mM EDTA. Blood samples were then
582 blocked with anti-CD32 (BD Pharmingen Cat. 550270) antibody for 15 min at 4°C. After blocking,
583 the cells were labeled; CD45 Alexa flour 700 (Biolegend Cat. 202218), CD161 APC (Biolegend
584 Cat. 205606), CD45R PE Cy7 (eBioscience Cat. 25-0460-82), His48 FITC (eBioscience Cat. 11-
585 0570-82), CD43 PE (Biolegend Cat. 202812), and CD3 VioGreen (Miltenyi Biotec Cat. 130-119-
586 125) (81). Samples were incubated 1 hr at 4°C in the dark. To prepare the lung samples for flow
587 cytometry, the lung homogenate was filtered through a 70 µm cell strainer (BioDesign Cell
588 MicroSives Cat. N70R). The suspension was centrifuged at 1,000 x *g* for 5 min. The pellet was
589 resuspended in Pharmlyse buffer and incubated at 37°C for 2 min. After incubation, the cells were
590 centrifuged at 1,000 x *g* for 5 min, lysis buffer removed, and cells were blocked and labeled with
591 antibody as described for blood samples. Both blood and lung samples were centrifuged at 1,000
592 x *g* for 5 min and the pellets were resuspended in 0.4% paraformaldehyde and stored overnight at
593 4°C. Samples were washed with 1x PBS+5mM EDTA+1%FBS and resuspended in 1x PBS+5mM

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

594 EDTA+1%FBS for analysis. Cell samples were analyzed on a LSR Fortessa and samples were
595 gated and analyzed using FlowJo v10.
596 **Cytokine analysis.** Lung homogenates were centrifuged at 19,000× *g* for 4 min and the resulting
597 supernatant was removed and stored at −80 °C until further analysis. Lung supernatant and serum
598 cytokines were measured using a ProcartaPlex Multiplex Immunoassay kit: Th Complete 14-Plex
599 Rat ProcartaPlex Panel (Thermo Fisher Scientific Cat. EPX140-30120-901) per the
600 manufacturer's instructions. Cytokines with bead counts less than 35 were invalidated.
601 **Generation of correlograms.** Correlograms were created using R Studio software. Pearson
602 correlation coefficients were calculated between each set of variables listed in the master table and
603 then illustrated in the representative plot for each vaccine route and timepoint.
604
605 **Statistical analysis.** GraphPad Prizm 7 was used to analyze the data. The minimum biological
606 replicates for the challenge studies were three for MVC control group and four rats per vaccinated
607 groups. For statistical comparisons between vaccinated groups and the MVC control group over
608 the entire course of the infection, two-way analysis of variance (ANOVA) was used with Dunnett's
609 post hoc test. One-way ANOVA was used for comparison between vaccinated groups and MVC
610 for an individual day or timepoint with Dunnett's post hoc test. Kruskal-Wallis test with Dunnett's
611 post hoc test compared between groups for mucosal IgA comparisons. ROUT test was used to
612 identify any potential outliers during cytokine analysis of the lung.
613 **Data availability.** Data requests for figures provided can be addressed to the corresponding author.
614 **Ethics statement.** This challenge study was performed in accordance with our approved protocol
615 by West Virginia University Institutional Animal Care and Use Committee (IACUC) protocol
616 1811019148.6.

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

617 **ACKNOWLEDGMENTS**

618 The work performed in this project was supported by the Vaccine Development Center at
619 WVU-HSC through a Research Challenge Grant no. HEPC.dsr.18.6 from the Division of Science
620 and Research, WV Higher Education Policy Commission. The project was also supported by NIH
621 R01AI137155 (F.H.D). Part of the project was also supported by CDC Contract Broad Agency
622 Announcement (BAA) 75D301-19-R-67835. Flow cytometry experiments were performed in the
623 West Virginia University Flow Cytometry Core Facility, which is supported by the National
624 Institutes of Health equipment grant number S10OD016165 and the Institutional Development
625 Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes
626 of Health under grant numbers P30GM103488 (CoBRE) and P20GM103434 (INBRE).

627 JMH and GJB performed vaccination and bacterial challenge. JMH and GJB monitored
628 rats by whole body plethysmography. All authors participated in the animal experiments. JMH and
629 TYW prepared flow cytometry samples. JMH and MAW performed cytokine analysis. JMH
630 performed ELISA assays. MAD constructed correlograms. JMH, MB, and FHD contributed to
631 experimental design. JMH wrote manuscript with critical revisions from all authors.

632 The authors would also like to thank Jacqueline Karakiozis, Brice Hickey, and Mary
633 Tomago-Chesney (Pathology/Histology Core Facility) for the preparation of lung slides and
634 staining lung slides with H&E for scoring analysis. The authors would also like to thank Dr.
635 Kathleen Brundage (WVU Flow Cytometry & Single Cell Core Facility) for assisting in flow
636 cytometry and equipment instruction.

637 The authors would also like to acknowledge that figures S1 and S8 were created with
638 BioRender.com

639

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

640 **References**

641 1. Bordet J, Gengou O. 1906. Le Microbe de la Coqueluche. Les Ann l'Institut Pasteur
642 20:731–741.
643 2. Mattoo S, Cherry JD. 2005. Molecular pathogenesis, epidemiology, and clinical
644 manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella*
645 subspecies. Clin Microbiol Rev 18:326–382.
646 3. Melvin JA, Scheller E V., Miller JF, Cotter PA. 2014. *Bordetella pertussis* pathogenesis:
647 Current and future challenges. Nat Rev Microbiol2014/03/13. 12:274–288.
648 4. Kapil P, Merkel TJ. 2019. Pertussis vaccines and protective immunity. Curr Opin
649 Immunol 59:72–78.
650 5. CDC, Ncird. Immunology and Vaccine-Preventable Diseases – Pink Book – Pertussis.
651 6. Pittman M. 1991. History of the development of pertussis vaccine. Dev Biol
652 Stand1991/01/01. 73:13–29.
653 7. Hill Elam-Evans LD, Yankey D, Singleton JA, Kang Y. HA. 2017. Vaccination Coverage
654 Among Children Aged 19–35 Months — United States, 2017. MMWR Morb Mortal
655 Wkly Rep 2018 1123–1128.
656 8. Yeung KHT, Duclos P, Nelson EAS, Hutubessy RCW. 2017. An update of the global
657 burden of pertussis in children younger than 5 years: a modelling study. Lancet Infect Dis
658 17:974–980.
659 9. Klein NP, Bartlett J, Fireman B, Baxter R. 2016. Waning Tdap Effectiveness in
660 Adolescents. Pediatrics 137:e20153326–e20153326.
661 10. Klein NP, Bartlett J, Rowhani-Rahbar A, Fireman B, Baxter R. 2012. Waning Protection
662 after Fifth Dose of Acellular Pertussis Vaccine in Children. N Engl J Med 367:1012–

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

663 1019.

664 11. Klein NP, Bartlett J, Fireman B, Rowhani-Rahbar A, Baxter R. 2013. Comparative
665 Effectiveness of Acellular Versus Whole-Cell Pertussis Vaccines in Teenagers. Pediatrics
666 131:e1716–e1722.

667 12. Althouse BM, Scarpino S V. 2015. Asymptomatic transmission and the resurgence of
668 *Bordetella pertussis*. BMC Med 13:146.

669 13. Templeton KE, Scheltinga SA, van der Zee A, Diederen BMW, van Kruijssen AM,
670 Goossens H, Kuijper E, Claas ECJ. 2003. Evaluation of real-time PCR for detection of
671 and discrimination between *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella*
672 *holmesii* for clinical diagnosis. J Clin Microbiol 41:4121–6.

673 14. Warfel JM, Zimmerman LI, Merkel TJ. 2014. Acellular pertussis vaccines protect against
674 disease but fail to prevent infection and transmission ina nonhuman primate model. Proc
675 Natl Acad Sci U S A 111:787–792.

676 15. Wendelboe AM, Van Rie A, Salmaso S, Englund JA. 2005. Duration of Immunity Against
677 Pertussis After Natural Infection or Vaccination. Pediatr Infect Dis J 24:S58–S61.

678 16. Wilk MM, Misiak A, McManus RM, Allen AC, Lynch MA, Mills KHG. 2017. Lung
679 CD4 Tissue-Resident Memory T Cells Mediate Adaptive Immunity Induced by Previous
680 Infection of Mice with *Bordetella pertussis*. J Immunol 199:233–243.

681 17. Chen K, Magri G, Grasset EK, Cerutti A. 2020. Rethinking mucosal antibody responses:
682 IgM, IgG and IgD join IgA. Nat Rev Immunol. Nature Research.

683 18. Goodman YE, Wort AJ, Jackson FL. 1981. Enzyme-linked immunosorbent assay for
684 detection of pertussis immunoglobulin A in nasopharyngeal secretions as an indicator of
685 recent infection. J Clin Microbiol 13:286–292.

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

686 19. Tuomanen EI, Zapiain LA, Galvan P, Hewlett EL. 1984. Characterization of antibody
687 inhibiting adherence of *Bordetella pertussis* to human respiratory epithelial cells. J Clin
688 Microbiol 20:167.
689 20. Hellwig SMM, Van Spriel AB, Schellekens JFP, Mooi FR, Van de Winkel JGJ. 2001.
690 Immunoglobulin A-mediated protection against *Bordetella pertussis* infection. Infect
691 Immun 69:4846–4850.
692 21. Thomas MG, Redhead K, Lambert HP. 1989. Human Serum Antibody Responses to
693 *Bordetella pertussis* Infection and Pertussis Vaccination. J Infect Dis 159:211–218.
694 22. Marcellini V, Piano Mortari E, Fedele G, Gesualdo F, Pandolfi E, Midulla F, Leone P,
695 Stefanelli P, Tozzi AE, Carsetti R. 2017. Protection against Pertussis in Humans
696 Correlates to Elevated Serum Antibodies and Memory B Cells. Front Immunol 8:1158.
697 23. Mills KH, Barnard A, Watkins J, Redhead K. 1993. Cell-mediated immunity to *Bordetella*
698 *pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model.
699 Infect Immun 61:399–410.
700 24. Dunne A, Ross PJ, Pospisilova E, Masin J, Meaney A, Sutton CE, Iwakura Y, Tschopp J,
701 Sebo P, Mills KHG. 2010. Inflammasome Activation by Adenylate Cyclase Toxin Directs
702 Th17 Responses and Protection against *Bordetella pertussis*. J Immunol 185:1711–1719.
703 25. Warfel JM, Merkel TJ. 2013. *Bordetella pertussis* infection induces a mucosal IL-17
704 response and long-lived Th17 and Th1 immune memory cells in nonhuman primates.
705 Mucosal Immunol 6:787–796.
706 26. Solans L, Locht C. 2019. The role of mucosal immunity in pertussis. Front Immunol.
707 Frontiers Media S.A.
708 27. Boehm DT, Wolf MA, Hall JM, Wong TY, Sen-Kilic E, Basinger HD, Dziadowicz SA,

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

709 Gutierrez M de la P, Blackwood CB, Bradford SD, Begley KA, Witt WT, Varney ME,
710 Barbier M, Damron FH. 2019. Intranasal acellular pertussis vaccine provides mucosal
711 immunity and protects mice from *Bordetella pertussis*. npj Vaccines 4.
712 28. Wolf MA, Boehm DT, DeJong MA, Wong TY, Sen-Kilic E, Hall JM, Blackwood CB,
713 Weaver KL, Kelly CO, Kisamore CA, Bitzer GJ, Bevere JR, Barbier M, Damron FH.
714 2020. Intranasal immunization with acellular pertussis vaccines results in long-term
715 immunity to *Bordetella pertussis* in mice . Infect Immun IAI.00607-20.
716 29. Maurer H, Höfler K, Hilbe W, Huber E. 1979. Preliminary findings with oral whooping
717 cough vaccination in young infants. Wien Hlin Wochenschr.
718 30. Baumann E, Binder BR, Falk W, Huber EG, Kurz R, Rosanelli K. 1985. Development and
719 clinical use of an oral heat-inactivated whole cell pertussis vaccine. Dev Biol Stand
720 61:511–6.
721 31. Guzman CA, Brownlie RM, Kadurugamuwa J, Walker MJ, Timmis KN. 1991. Antibody
722 responses in the lungs of mice following oral immunization with *Salmonella typhimurium*
723 *aroA* and invasive *Escherichia coli* strains expressing the filamentous hemagglutinin of
724 *Bordetella pertussis*. Infect Immun 59:4391–4397.
725 32. Lim A, Ng JKW, Locht C, Alonso S. 2014. Protective role of adenylate cyclase in the
726 context of a live pertussis vaccine candidate. Microbes Infect 16:51–60.
727 33. Skerry CM, Mahon BP. 2011. A live, attenuated *Bordetella pertussis* vaccine provides
728 long-term protection against virulent challenge in a murine model. Clin Vaccine
729 Immunol2010/12/08. 18:187–193.
730 34. Thorstensson R, Trollfors B, Al-Tawil N, Jahnmatz M, Bergström J, Ljungman M, Törner
731 A, Wehlin L, Van Broekhoven A, Bosman F, Debrie AS, Mielcarek N, Locht C. 2014. A

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

732 phase I clinical study of a live attenuated *Bordetella pertussis* vaccine - BPZE1; a single
733 centre, double-blind, placebo-controlled, dose-escalating study of BPZE1 given
734 intranasally to healthy adult male volunteers. PLoS One 9:e83449.
735 35. Lin A, Apostolovic D, Jahnmatz M, Liang F, Ols S, Tecleab T, Wu C, van Hage M,
736 Solovay K, Rubin K, Locht C, Thorstensson R, Thalen M, Loré K. 2020. Live attenuated
737 pertussis vaccine BPZE1 induces a broad antibody response in humans. J Clin Invest
738 130:2332–2346.
739 36. Hornibrook JW, Ashburn LL. 1939. A Study of Experimental Pertussis in the Young Rat.
740 Public Heal Reports 54:439.
741 37. Woods DE, Franklin R, Cryz SJ, Ganss M, Peppler M, Ewanowich C. 1989. Development
742 of a rat model for respiratory infection with *Bordetella pertussis*. Infect Immun 57:1018–
743 1024.
744 38. Hall E, Parton R, Wardlaw AC. 1994. Cough production, leucocytosis and serology of rats
745 infected intrabronchially with *Bordetella pertussis*. J Med Microbiol 40:205–213.
746 39. Parton R, Hall E, Wardlaw AC. 1994. Responses to *Bordetella pertussis* mutant strains
747 and to vaccination in the coughing rat model of pertussis. J Med Microbiol 40:307–312.
748 40. Hall E, Parton R, Wardlaw AC. 1997. Differences in coughing and other responses to
749 intrabronchial infection with *Bordetella pertussis* among strains of rats. Infect Immun
750 65:4711–4717.
751 41. Hall E, Parton R, Wardlaw AC. 1998. Responses to acellular pertussis vaccines and
752 component antigens in a coughing-rat model of pertussis. Vaccine 16:1595–603.
753 42. Hall E, Parton R, Wardlaw AC. 1999. Time-course of infection and responses in a
754 coughing rat model of pertussis. J Med Microbiol 48:95–98.

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

755 43. Hall JM, Kang J, Kenney SM, Wong TY, Bitzer GJ, Kelly CO, Kisamore CA, Boehm DT,
756 DeJong MA, Allison M, Sen-Kilic E, Horspool AM, Bevere JR, Barbier M, Heath
757 Damron F. 2021. Re-investigating the coughing rat model of pertussis to understand
758 *Bordetella pertussis* pathogenesis . bioRxiv 2021.04.02.438291.
759 44. Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, Lavelle EC, McLoughlin
760 RM, Mills KHG. 2013. Relative Contribution of Th1 and Th17 Cells in Adaptive
761 Immunity to *Bordetella pertussis*: Towards the Rational Design of an Improved Acellular
762 Pertussis Vaccine. PLoS Pathog 9:e1003264.
763 45. Ryan M, Murphy G, Ryan E, Nilsson L, Shackley F, Gothefors L, Øymar K, Miller E,
764 Storsaeter J, Mills KH. 1998. Distinct T-cell subtypes induced with whole cell and
765 acellular pertussis vaccines in children. Immunology 93:1–10.
766 46. Ausiello CM, Urbani F, la Sala A, Lande R, Cassone A. 1997. Vaccine- and antigen-
767 dependent type 1 and type 2 cytokine induction after primary vaccination of infants with
768 whole-cell or acellular pertussis vaccines. Infect Immun 65:2168–2174.
769 47. Mahon BP, Sheahan BJ, Griffin F, Murphy G, Mills KH. 1997. Atypical disease after
770 *Bordetella pertussis* respiratory infection of mice with targeted disruptions of interferon-
771 gamma receptor or immunoglobulin mu chain genes. J Exp Med 186:1843–51.
772 48. Barbic J, Leef MF, Burns DL, Shahin RD. 1997. Role of gamma interferon in natural
773 clearance of *Bordetella pertussis* infection. Infect Immun 65:4904–4908.
774 49. Mills KHG, Ryan M, Ryan E, Mahon BP. 1998. A murine model in which protection
775 correlates with pertussis vaccine efficacy in children reveals complementary roles for
776 humoral and cell- mediated immunity in protection against *Bordetella pertussis*. Infect
777 Immun 66:594–602.

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

778 50. Redhead K, Watkins J, Barnard A, Mills KHG. 1993. Effective immunization against
779 *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell-
780 mediated immunity. Infect Immun 61:3190–3198.
781 51. Sawal M, Cohen M, Irazuzta JE, Kumar R, Kirton C, Brundler M-A, Evans CA, Wilson
782 JA, Raffeeq P, Azaz A, Rotta AT, Vora A, Vohra A, Abboud P, Mirkin LD, Cooper M,
783 Dishop MK, Graf JM, Petros A, Klonin H. 2009. Fulminant pertussis: A multi-center
784 study with new insights into the clinico-pathological mechanisms. Pediatr Pulmonol
785 44:970–980.
786 52. Paddock CD, Sanden GN, Cherry JD, Gal AA, Langston C, Tatti KM, Wu K, Goldsmith
787 CS, Greer PW, Montague JL, Eliason MT, Holman RC, Guarner J, Shieh W, Zaki SR.
788 2008. Pathology and Pathogenesis of Fatal *Bordetella pertussis* Infection in Infants. Clin
789 Infect Dis 47:328–338.
790 53. Morse SI, Morse JH. 1976. Isolation and properties of the leukocytosis- and
791 lymphocytosis- promoting factor of *Bordetella pertussis*. J Exp Med 143:1483–1502.
792 54. MORSE SI. 1965. STUDIES ON THE LYMPHOCYTOSIS INDUCED IN MICE BY
793 *BORDETELLA PERTUSSIS*. J Exp Med 121:49–68.
794 55. Morse SI, Riester SK. 1967. Studies on the leukocytosis and lymphocytosis induced by
795 *Bordetella pertussis*. I. Radioautographic analysis of the circulating cells in mice
796 undergoing pertussis-induced hyperleukocytosis. J Exp Med 125:401–8.
797 56. Heininger U, Klich K, Stehr K, Cherry JD. 1997. Clinical findings in *Bordetella pertussis*
798 infections: results of a prospective multicenter surveillance study. Pediatrics 100:E10.
799 57. Blackwood CB, Sen-Kilic E, Boehm DT, Hall JM, Varney ME, Wong TY, Bradford SD,
800 Bevere JR, Witt WT, Damron FH, Barbier M. 2020. Innate and adaptive immune

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

801 responses against *Bordetella pertussis* and *Pseudomonas aeruginosa* in a murine model of
802 mucosal vaccination against respiratory infection. Vaccines 8:1–21.
803 58. Correlogram. https://www.r-graph-gallery.com/correlogram.html
804 59. Bewick V, Cheek L, Ball J. 2003. Statistics review 7: Correlation and regression. Crit
805 Care. BioMed Central.
806 60. Althouse BM, Scarpino S V. 2015. Asymptomatic transmission and the resurgence of
807 *Bordetella pertussis* https://doi.org/10.1186/s12916-015-0382-8.
808 61. Klein NP, Bartlett J, Fireman B, Aukes L, Buck PO, Krishnarajah G, Baxter R. 2017.
809 Waning protection following 5 doses of a 3-component diphtheria, tetanus, and acellular
810 pertussis vaccine. Vaccine 35:3395–3400.
811 62. Nakamura K, Shinoda N, Hiramatsu Y, Ohnishi S, Kamitani S, Ogura Y, Hayashi T,
812 Horiguchi Y. 2019. BspR/BtrA, an Anti-σ Factor, Regulates the Ability of *Bordetella*
813 *bronchiseptica* To Cause Cough in Rats. mSphere https://doi.org/10.1128/msphere.00093-
814 19.
815 63. Boehm DT, Hall JM, Wong TY, DiVenere A, Sen-Kilic E, Bevere JR, Bradford SD,
816 Blackwood CB, Elkins C, DeRoos KA, Gray MC, Cooper CG, Varney ME, Maynard JA,
817 Hewlett EL, Barbier M, Damron FH. 2018. Evaluation of adenylate cyclase toxoid antigen
818 in acellular pertussis vaccines using a *Bordetella pertussis* challenge model in mice. Infect
819 Immun IAI.00857-17.
820 64. Shi W, Kou Y, Jiang H, Gao F, Kong W, Su W, Xu F, Jiang C. 2018. Novel intranasal
821 pertussis vaccine based on bacterium-like particles as a mucosal adjuvant. Immunol Lett
822 198:26–32.
823 65. Allen AC, Wilk MM, Misiak A, Borkner L, Murphy D, Mills KHG. 2018. Sustained

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

824 protective immunity against *Bordetella pertussis* nasal colonization by intranasal
825 immunization with a vaccine-adjuvant combination that induces IL-17-secreting T RM
826 cells. Mucosal Immunol 11:1763–1776.
827 66. Ryan EJ, Mcneela E, Murphy GA, Stewart H, O'Hagan D, Pizza M, Rappuoli R, Mills
828 KHG. 1999. Mutants of *Escherichia coli* heat-labile toxin act as effective mucosal
829 adjuvants for nasal delivery of an acellular pertussis vaccine: Differential effects of the
830 nontoxic AB complex and enzyme activity on Th1 and Th2 cells. Infect Immun 67:6270–
831 6280.
832 67. Locht C, Papin JF, Lecher S, Debrie A-S, Thalen M, Solovay K, Rubin K, Mielcarek N.
833 2017. Live Attenuated Pertussis Vaccine BPZE1 Protects Baboons Against *Bordetella*
834 *pertussis* Disease and Infection. J Infect Dis2017/05/23. 216:117–124.
835 68. Solans L, Debrie A-S, Borkner L, Aguiló N, Thiriard A, Coutte L, Uranga S, Trottein F,
836 Martín C, Mills KHG, Locht C. 2018. IL-17-dependent SIgA-mediated protection against
837 nasal *Bordetella pertussis* infection by live attenuated BPZE1 vaccine. Mucosal Immunol
838 11:1753–1762.
839 69. Lin A, Apostolovic D, Jahnmatz M, Liang F, Ols S, Tecleab T, Wu C, van Hage M,
840 Solovay K, Rubin K, Locht C, Thorstensson R, Thalen M, Loré K. 2020. Live attenuated
841 pertussis vaccine BPZE1 induces a broad antibody response in humans. J Clin Invest 130.
842 70. Baumann E, Binder BR, Falk W. 1985. Development and clinical use of an oral heat-
843 inactivated whole cell pertussis vaccine. Dev Biol Stand VOL. 61:511–516.
844 71. Strugnell R, Dougan G, Chatfield S, Charles I, Fairweather N, Tite J, Li JL, Beesley J,
845 Roberts M. 1992. Characterization of a *Salmonella typhimurium* aro vaccine strain
846 expressing the P.69 antigen of *Bordetella pertussis*. Infect Immun 60.

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

847 72. Molina NC, Parker CD. 1990. Murine antibody response to oral infection with live aroA
848 recombinant *Salmonella dublin* vaccine strains expressing filamentous hemagglutinin
849 antigen from *Bordetella pertussis*. Infect Immun 58.
850 73. Plotkin SA. 2010. Correlates of protection induced by vaccination. Clin Vaccine
851 Immunol. American Society for Microbiology.
852 74. Murphy TV, Slade BA, Broder KR, Kretsinger K, Tiwari T, Joyce PM I, JK, Brown K
853 MJAC on IP, Prevention. C for DC and. 2008. Prevention of Pertussis, Tetanus, and
854 Diphtheria Among Pregnant and Postpartum Women and their Infants Recommendations
855 of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Reports
856 1–51.
857 75. Frizzell H, Woodrow KA. 2020. Biomaterial Approaches for Understanding and
858 Overcoming Immunological Barriers to Effective Oral Vaccinations. Adv Funct Mater
859 30:1907170.
860 76. Azizi A, Kumar A, Diaz-Mitoma F, Mestecky J. 2010. Enhancing oral vaccine potency by
861 targeting intestinal M cells. PLoS Pathog. Public Library of Science.
862 77. Kilgore PE, Salim AM, Zervos MJ, Schmitt H-J. 2016. Pertussis: Microbiology, Disease,
863 Treatment, and Prevention. Clin Microbiol Rev 29:449–86.
864 78. Scheller E V., Cotter PA. 2015. *Bordetella* filamentous hemagglutinin and fimbriae:
865 critical adhesins with unrealized vaccine potential. Pathog Dis 73:ftv079.
866 79. Stainer DW, Scholte MJ. 1970. A Simple Chemically Defined Medium for the Production
867 of Phase I *Bordetella pertussis*. J Gen Microbiol1970/10/01. 63:211–220.
868 80. Lomask J, Larson R. 2006. United States Patent USOO7104962B2. US 7,104,962 B2.
869 81. Barnett-Vanes A, Sharrock A, Birrell MA, Rankin S. 2016. A Single 9-Colour Flow

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

870 Cytometric Method to Characterise Major Leukocyte Populations in the Rat: Validation in
871 a Model of LPS-Induced Pulmonary Inflammation
872 https://doi.org/10.1371/journal.pone.0142520.

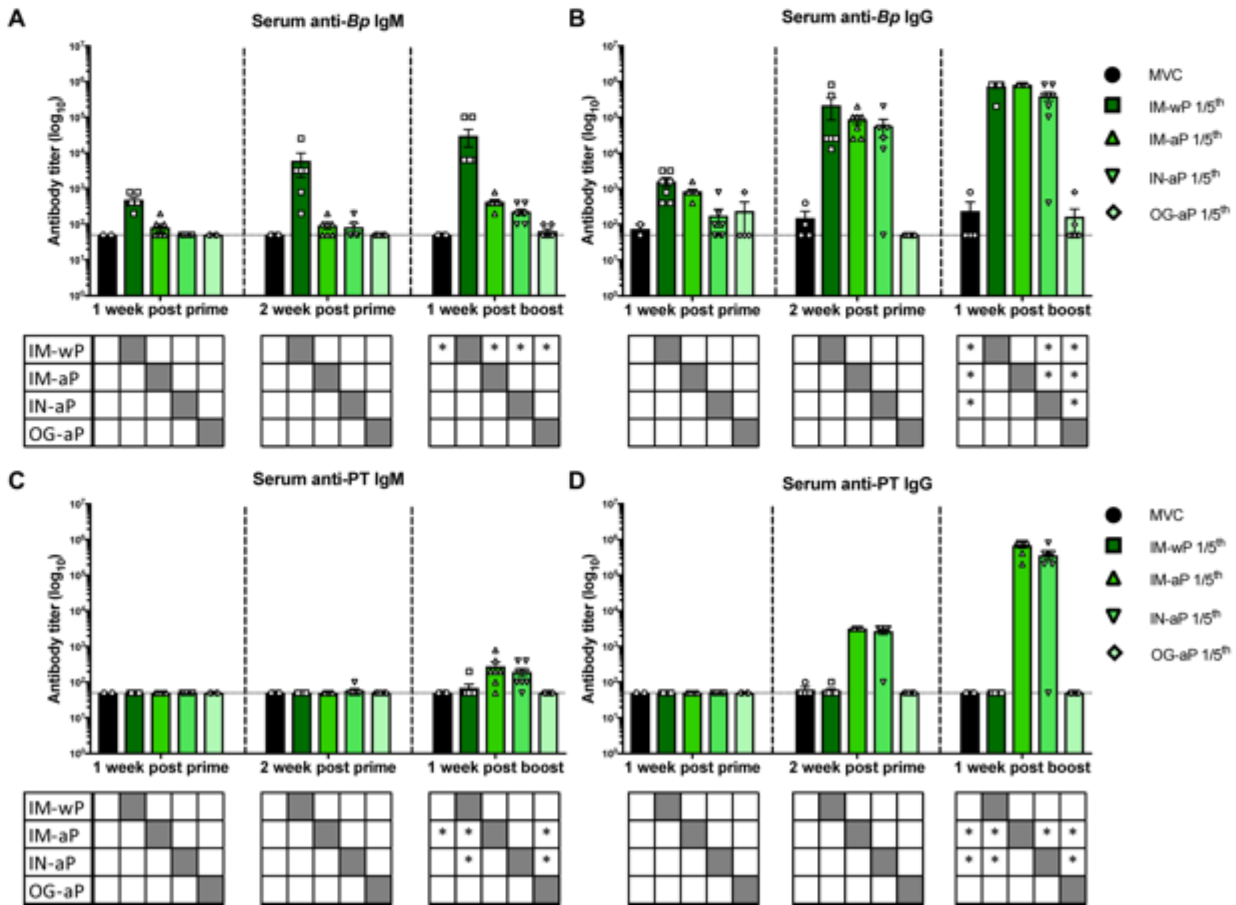
873

874 **FIGURES**

875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

893 **Figure 1**



894

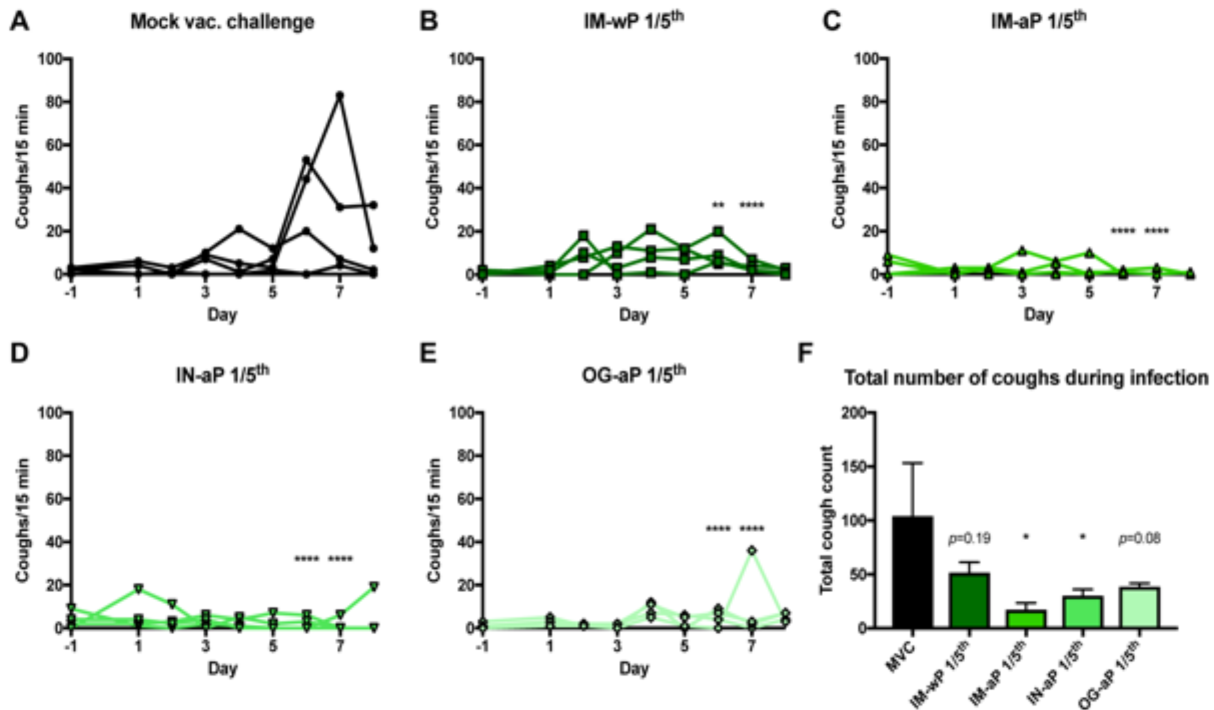
895 **FIG 1** IN booster vaccination induces systemic anti-Bp and anti-PT antibody titers. 1 and 2 weeks
896 post prime immunization and 1 week post boost blood was collected via saphenous vein, and anti
897 Bp and anti PT IgM (A-C) and IgG (B-D) specific antibodies were measured. Results are shown
898 on a log scale and as a mean ± SEM (n = 3-8). Dotted line represents the limit of detection.
899 *P < 0.05. (n = 4-8). P values were determined by two-way ANOVA with Dunnett's post hoc test
900 compared between groups. * under each graph annotates the significance between labeled group
901 under the y-axis and the group under the corresponding bar. Grayed out box annotates no stats
902 calculated.

903

904

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

905 **Figure 2**



906

907 **FIG 2** Intranasal and oral vaccination of acellular pertussis vaccine decreases cough of *B. pertussis*
908 infected rats. Coughs were counted every day of the nine-day infection using whole body
909 plethysmography. Coughs were counted for (A) mock vac. challenge rats, (B) IM-wP (C) IM-aP
910 (D) IN-aP, and (E) OG-aP, vaccinated and challenged rats. To assess any potential differences
911 between vaccine groups over the entire course of infection, (F) average total number of coughs for
912 each rat per group was compared. Results shown as mean ± SEM ($n = 3$-4). P values were
913 determined by two-way ANOVA with Dunnett's post hoc test and one-way ANOVA with Dunnett
914 post hoc test for total cough count, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$
915 compared to the mock vac. challenged control group.

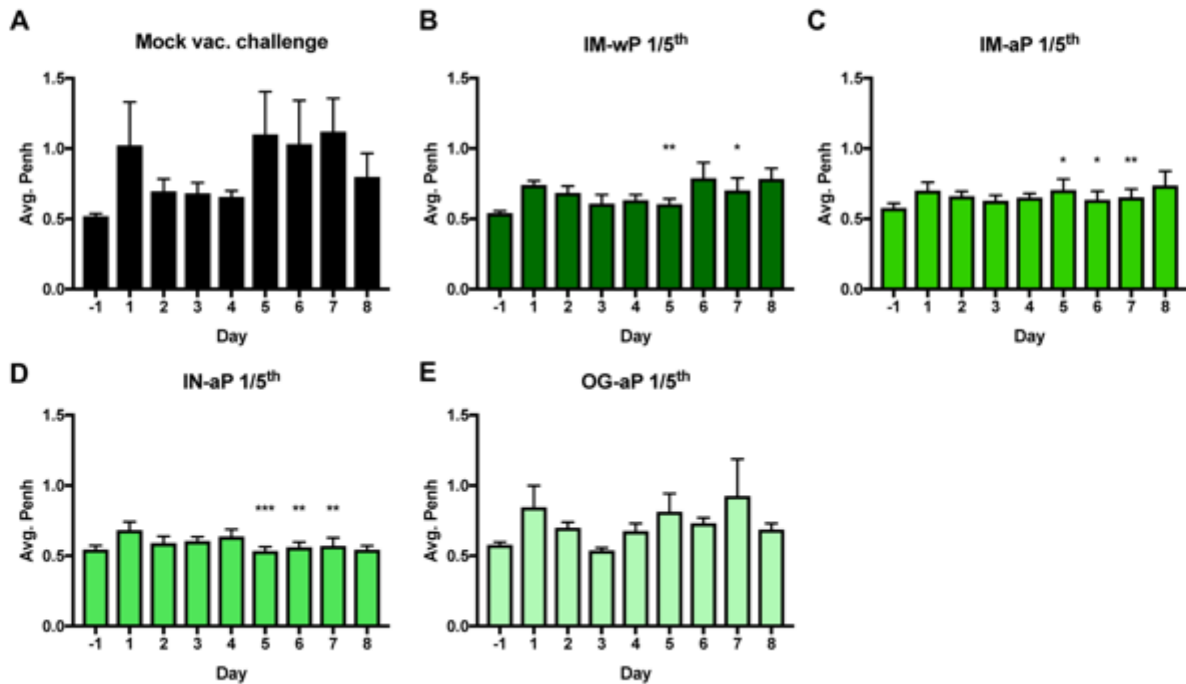
916

917

918

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

919 **Figure 3**



920

921 **FIG 3** Intranasal vaccination decreases pulmonary restriction of *Bordetella pertussis* infected rats.
922 Bronchiole restriction was measured over the course of infection by whole body plethysmography.
923 Bronchiole restriction was determined by the factor Penh for (A) mock vac. challenge rats, (B)
924 IM-wP (C) IM-aP (D) IN-aP, and (E) OG-aP vaccinated and challenged rats. Results shown as
925 mean ± SEM (n = 3-4). P values were determined by two-way ANOVA with Dunnett's post hoc
926 test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the mock vac. challenge group.

927

928

929

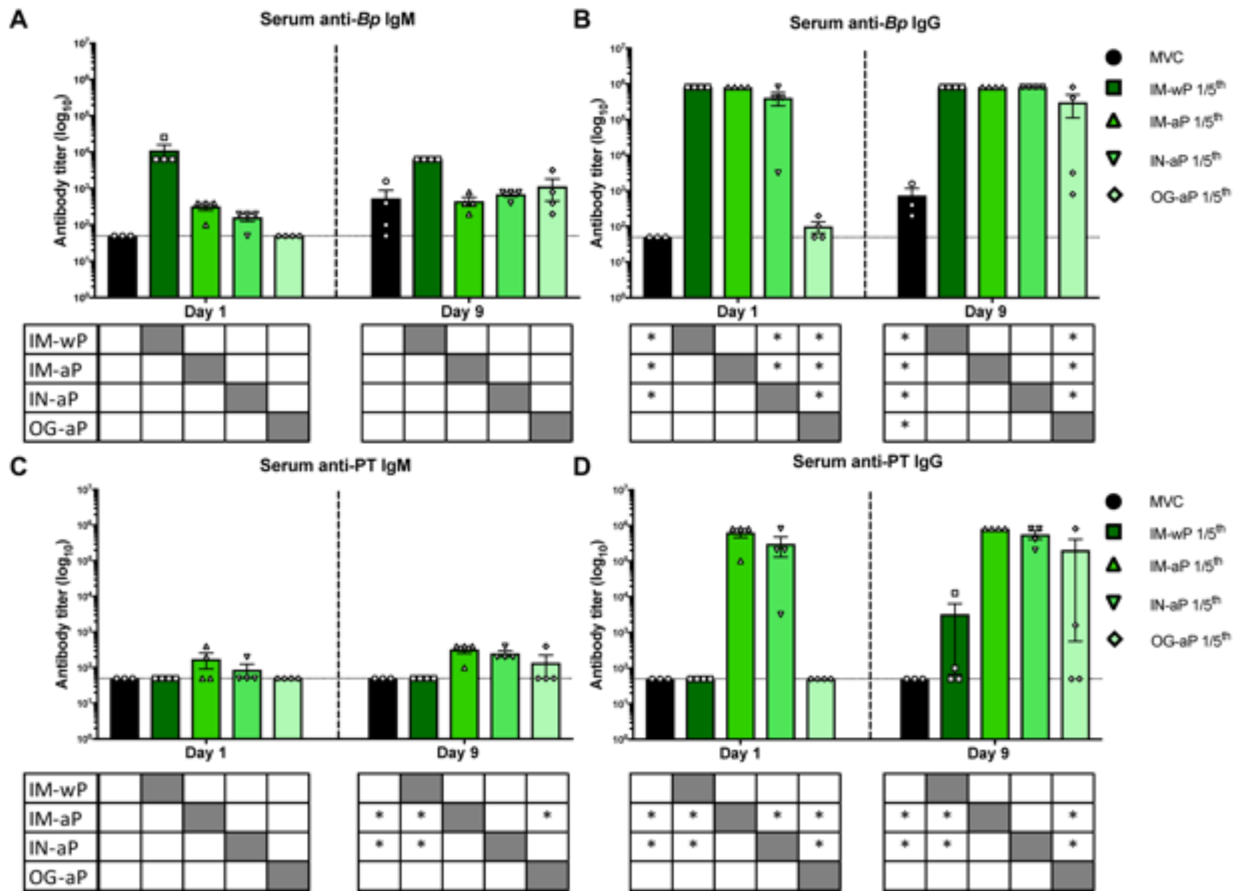
930

931

932

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

933 **Figure 4**



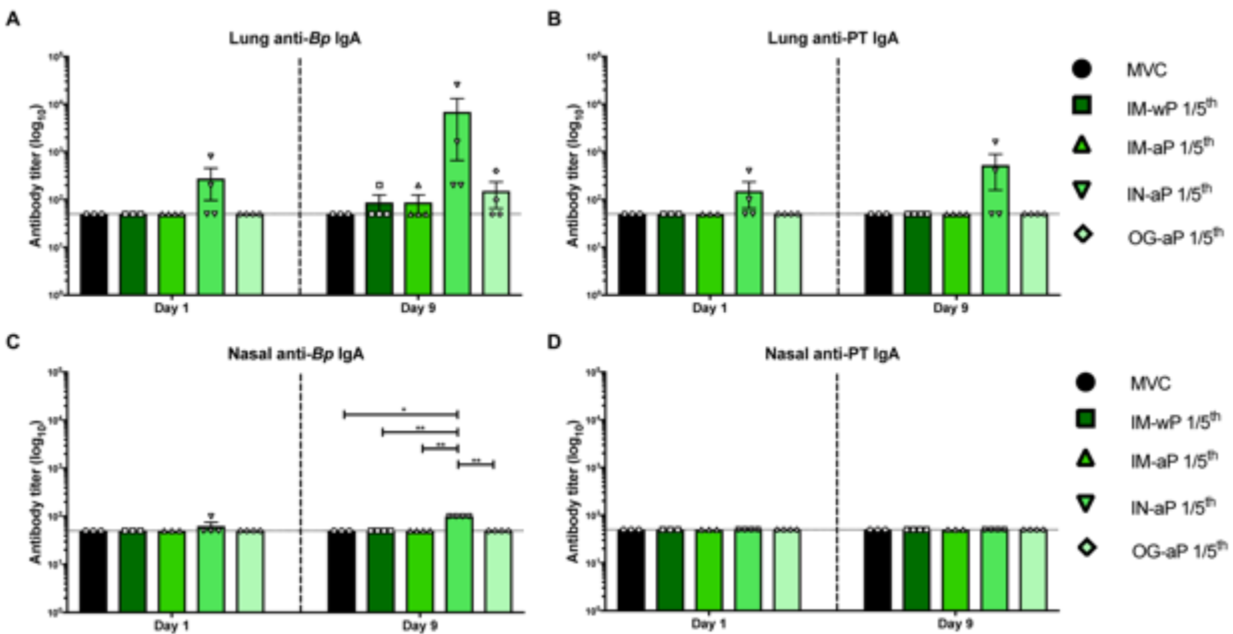
934

935 **FIG 4** Mucosal vaccination induces production of anti-*Bp* IgG, while IN immunization also
936 induces both anti-PT IgM and IgG antibodies. ELISAs were used to determine and compare the
937 induced serological responses from vaccinated and challenge rats in the serum. Both (A, C) IgM
938 and (B, D) IgG serum antibody titers from immunized and challenged rats were measured post
939 prime, boost, and challenge. Dotted line represents the limit of detection. Results are shown on a
940 log scale and as mean ± SEM, $*P < 0.05$ ($n = 4$). P values were determined by two-way ANOVA
941 with Dunnett's post hoc test compared between groups. * under each graph annotates the
942 significance between labeled group under the y-axis and the group under the corresponding bar.
943 Grayed out box annotates no stats calculated.

944

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

945 **Figure 5**

946


947 **FIG 5** Intranasal immunization elicits the production of anti-*Bp* IgA in the respiratory tract. ELISA
948 was used analyze antibodies in the (A, B) lung and (C, D) nasal cavity from lung homogenate
949 supernatant and PBS flushed through the nasal cavity from vaccinated and challenge rats at days
950 1 and 9 post challenge. IgA titers were determined against pertussis toxin and *B. pertussis*. Dotted
951 line represents the limit of detection. Results are shown on a log scale and as a mean ± SEM (n = 3-
952 4). **P < 0.01, ****P < 0.0001 *P* values were determined by Kruskal-Wallis test with Dunnett's
953 post hoc test compared between groups.

954

955

956

957

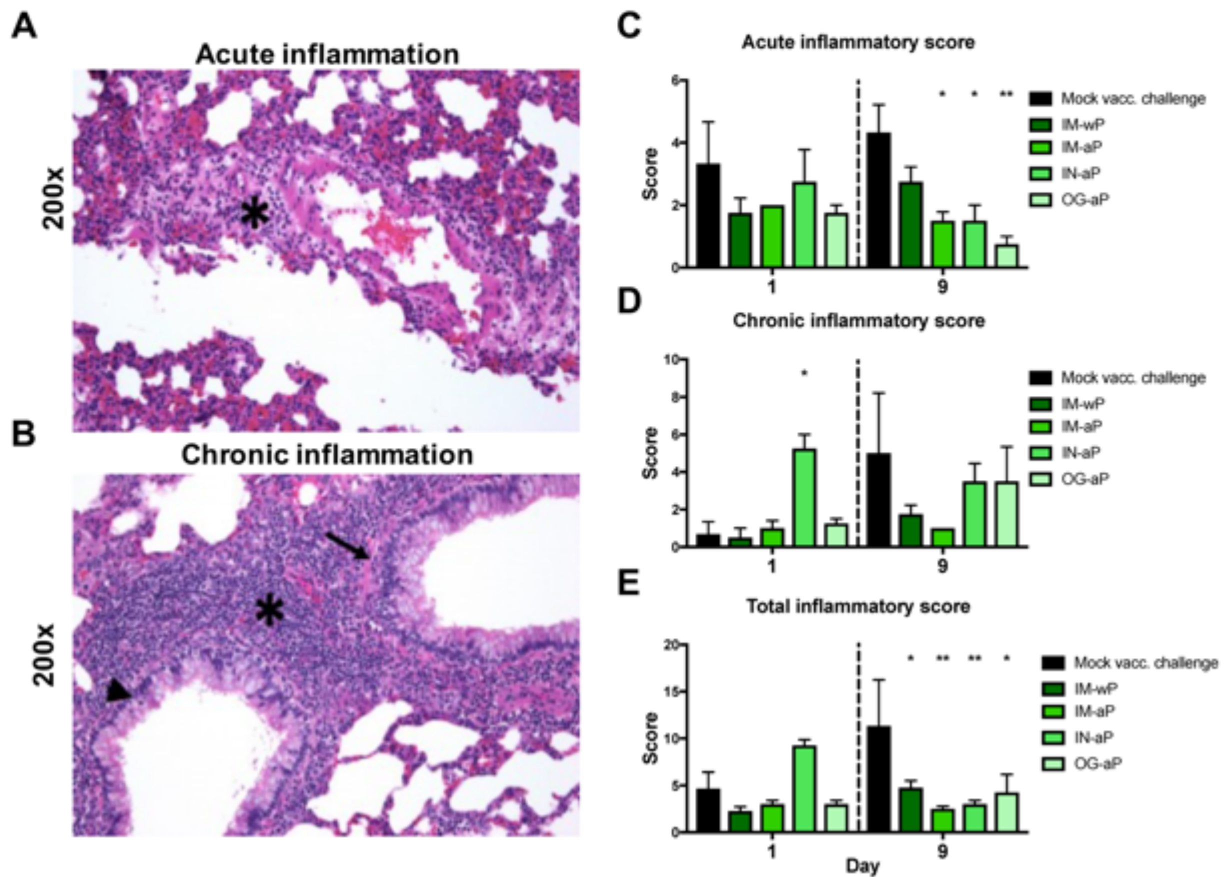
958

959

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

960

961 **Figure 6**



962

963 **FIG 6** Mucosal vaccination protects against acute and total inflammation in the lung of *Bp* infected
964 Sprague-Dawley rats. Post euthanasia, left lobe of the lung was excised, sectioned, and stained
965 with hematoxylin and eosin. Lung samples scores were based on standard qualitative toxicologic
966 scoring criteria (0 – none, 1 – minimal (rare), 2 – mild (slight), 3 – moderate, 4 – marked, 5 –
967 severe). (A) Representative image of acute inflammation of rat lung showing increased numbers
968 of neutrophils and edema surrounding blood vessel (asterisk). (B) Representative image of chronic
969 inflammation of the rat lung showing increased numbers of mononuclear cells surrounding
970 bronchioles (asterisk). Inflammatory cells are also present in the lamina propria (arrow) and
971 epithelium (arrowhead) of bronchioles. (C) Average acute inflammation scores of the lung are

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

972 detailed by the presence of neutrophils in the parenchyma, blood vessels, and the airways. (D)
973 Average chronic inflammation scores are distinguished by mononuclear infiltrates in the
974 parenchyma, blood vessels, and airway of the lung. (E) Total inflammatory score calculated by the
975 sum of the acute and chronic inflammation score of the lung. All scoring assessments were
976 determined with no knowledge of the groups. Results are shown as mean ± SEM (n = 3-4) P values
977 were determined by two-way ANOVA followed by Dunnett's comparison test, *$P < 0.05$,
978 **$P < 0.01$ compared to mock challenge.

979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 7

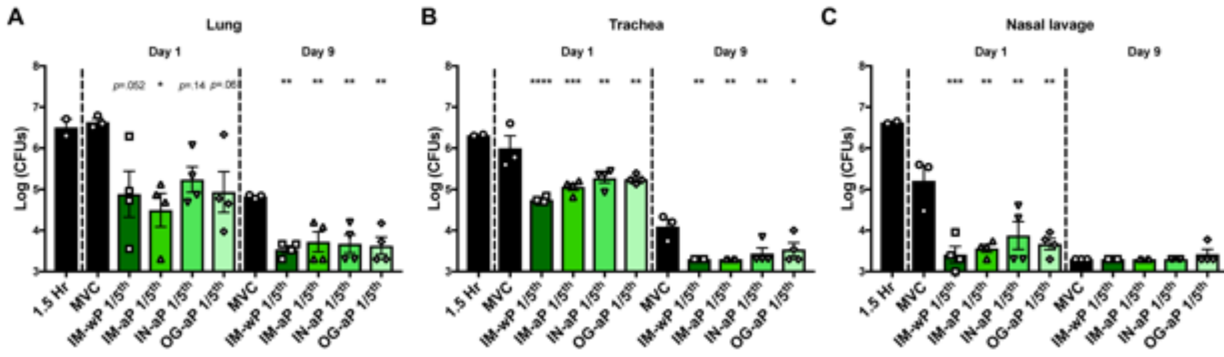
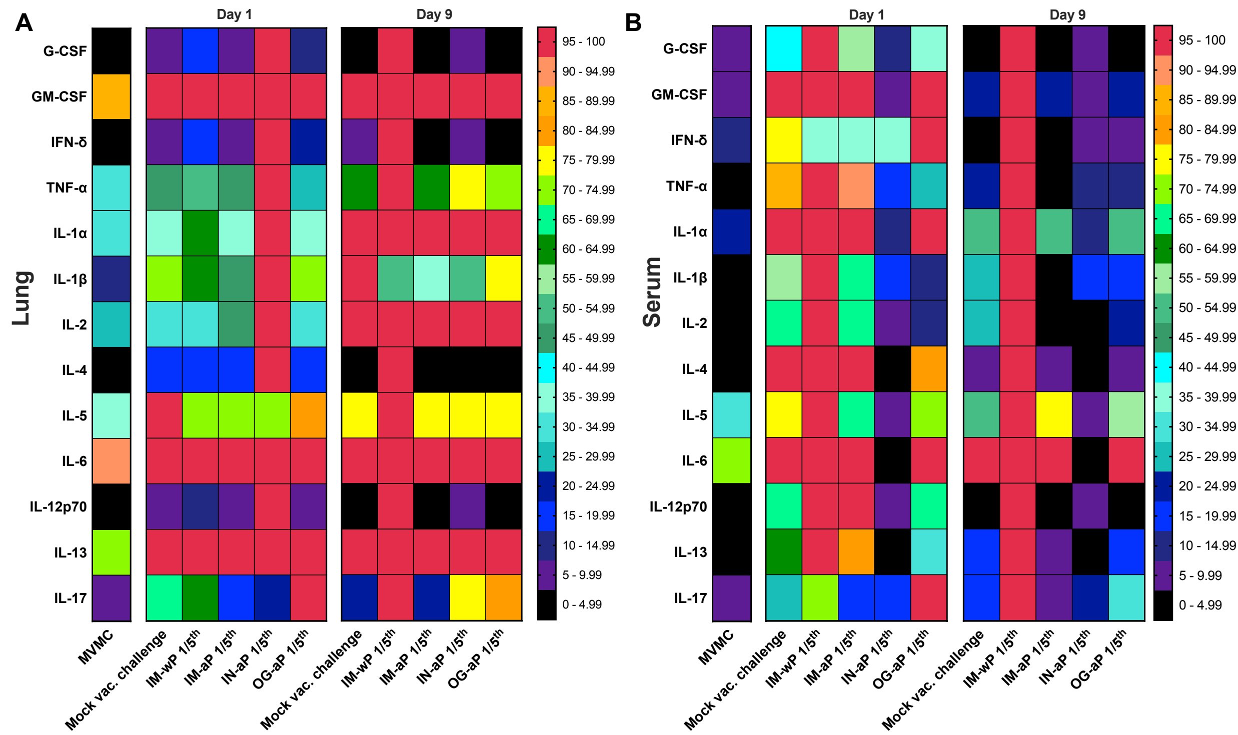


FIG 7 Oral and intranasal immunization decreased *B. pertussis* bacterial burden in the respiratory tract. Bacteria were quantified by serially diluted CFUs following vaccination and intranasal challenge. CFU counts were determined from (A) lung homogenate (B) trachea and (C) nasal lavage 1.5Hr, 1-, and 9-day post *B. pertussis* challenge. Results are shown as mean ± SEM ($n = 2$-4). P values were determined by one-way ANOVA with Dunnett's post hoc test, *$P < 0.05$, **$P < 0.01$, ***$P<0.001$, ****$P < 0.0001$ compared to mock vac. challenge group.

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1014 **Figure 8**



1015

1016 **FIG 8** Measurement of cytokines in the lung and serum at days 1 and 9 post infection. Heat map
1017 of the average percent cytokines normalized to the max cytokine measured in the (A) lung and (B)
1018 serum. MVMC (mock vaccinated mock challenge) cytokines are from rats in (Hall et al 2021). All
1019 statistical analysis comparing average cytokine values are in Fig S5-6.

1020

1021

1022

1023

1024

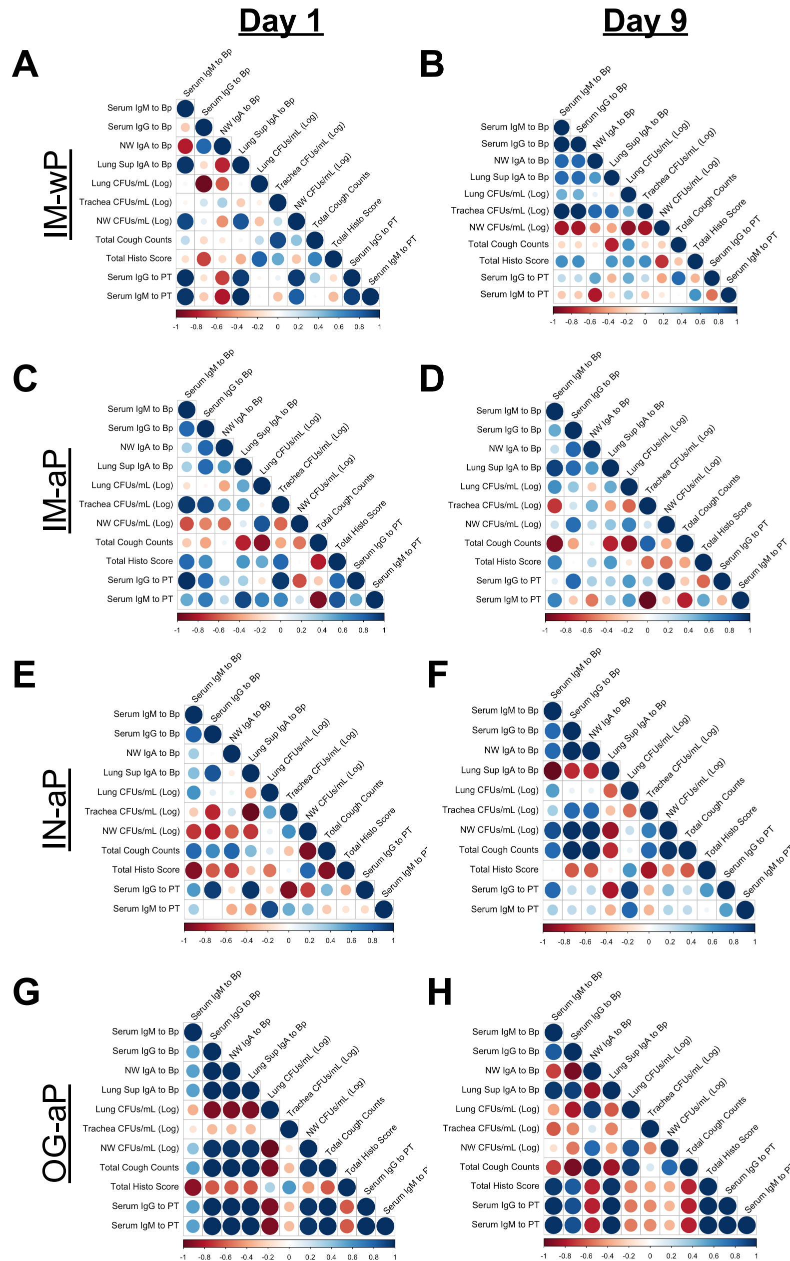
1025

1026

1027

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1028 **Figure 9**



1029

1030 **Fig 9** Systemic and mucosal anti-*Bp* and anti-PT antibodies correlate with observed protection.

1031 Correlograms were generated using the observed data for IM-wP (A-B), IM-aP (C-D), IN-aP (E-

1032 F), and OG-aP (G-H). Program R was used to make correlation graphs from raw data for both day

1033 1 and day 9 post-challenge. R^2 values were generated when generating the correlograms. Positive

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.21.449353; this version posted June 22, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1034 correlations are annotated by the blue circles, while the negative correlations are annotated by the
1035 red circles. The size of the circle annotates the strength of the correlation.
1036
1037